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AN ELECTRON MICROSCOPIC STUDY OF
AMOEBAE WITH PARTICULAR REFERENCE
TO INDUCED PINOCYTOSIS.

by

A.F. HAYWARD

VOLUME 1

TEXT

Ph.D. Thesis

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GENERAL INTRODUCTION

The fresh water amoebae, Amoeba proteus and Pelomyxa carolinensis have been the subject of extensive research especially in cell physiology and biochemistry. One of the phenomena for which they have provided a good deal of information is that known as "pinocytosis" - the ingestion of fluid by cells by invagination of the outer membrane to form intracellular droplets. The recent interest in pinocytosis has been stimulated to a large extent by electron microscopy but the electron microscopic study of amoebae has not been so thoroughly investigated as that of metazoan cells. Despite a number of papers on the detailed appearance of amoebae at least two of the cellular components well known from light microscopy have eluded absolute identification in the electron microscope. Pinocytosis in amoebae has not yet been fully explored with the higher resolution possible with this instrument.

This study includes some observations on the morphological changes produced by pinocytosis and as a result of the considerable number of micrographs of normal amoebae obtained enables a survey of normal structure to be made. To support some findings in normal Amoeba proteus, micrographs were prepared of centrifuged Pelomyxa carolinensis and are fully described.

Incidental and rather isolated findings associated with a bacterial contaminant of Amoeba proteus are also included.

The problem of pinocytosis

It has been known for many years that the fresh water amoeba Amoeba proteus responds to certain changes in its surrounding medium by ingesting discrete droplets of that medium. The first recorded induction of this phenomenon is in the work of Edwards (1925) who observed invaginations of the plasma membrane in amoebae exposed to dilute saline. Edwards interpreted the structures formed as food cups by analogy with the normal feeding mechanism of the organism. Some years later Lewis (1931) observed a process in amoeboid cultured cells which though differing morphologically from that seen by Edwards, also involved the ingestion of fluid in discrete membrane bound sacs. Cultured cells in accelerated cine micrographs, exhibit constant movement of the plasma membrane, thrown into a series of ruffles it falls back on to the cell surface to trap droplets of the medium. This type of activity appears to be normal among cultured cells and has been observed many times since Lewis's original experiments. On the suggestion of a colleague, Lewis called the process pinocytosis and this name has become accepted by most

workers for all the phenomena by which discrete droplets of fluid are drawn into cells.

Mast and Doyle (1934), aware of both Lewis's and Edwards' work showed that proteins introduced into the medium in which amoebae were growing induced the formation of channel-like structures invaginated from the plasma membrane and a series of small droplets formed at the end of the channels (diagram 2 is reproduced from Mast and Doyle 1934).

There was a pause in the observations on amoebae and cultured cells following the work of Mast and Doyle and no use of the term pinocytosis in cell physiology until the advent of successful biological electron microscopy. Palade (1953) in one of the earlier publications in this field suggested that some small droplets in the cytoplasm of capillary endothelium might be the result of pinocytosis. Since that time, a vast number of references to the possible or proved part taken by pinocytosis in the structure and physiology of cells has appeared.

The increasing emphasis on pinocytosis in metazoan cells has lead to a re-examination of the phenomenon in amoebae. It has been particularly fruitful to study the various types of substance which induce pinocytosis. Induction has been proved in metazoan

cells in only two cases. Barnett and Ball (1960) showed that the physiological action of insulin on adipose tissue is accompanied and possibly mediated by increased pinocytosis. Insulin also induces acceleration of pinocytosis in cultured HeLa cells though not in a reproducible manner (Paul and Pearson 1960, Paul, personal communication). Much of the work on amoebae, though by no means all, has come from the laboratory of Holter (1959) in particular from Chapman-Andresen (1963). In brief the phenomenon of pinocytosis is induced in amoebae by salts and proteins as well as amino acids, basic dyes, and colloidal heavy metal particles. The morphological picture varies with the inducer used and the effect is influenced by the concentration of solute (inducer), temperature and pH. It varies with the strain of amoeba and both with the quantity and type of food they have received.

In general, the reaction of an amoeba to contact with an inducer follows a pattern. The organism quickly stops the normal cytoplasmic streaming, withdraws the larger locomotory pseudopodia and "rounds off". It takes on a rosette-like shape with numerous small blunt processes over the whole surface. From the apex of such processes a channel passes down into the cytoplasm. Small vesicles break off from the channel end and after a

few minutes the whole channel breaks into short lengths. New channel formation continues for a period characteristic of the inducer but then ceases. The amoeba remains spherical. If the inducer is removed the amoeba slowly recovers its normal activity. If the inducer is still present a fresh burst of channel formation of less intensity follows after a period of quiescence. Throughout this period the contractile vacuole is large and paralysed i.e. it does not enter systole (see section 2). If the inducer is not removed, the amoeba continues bursts of channel activity of decreasing intensity at increasingly long time intervals and eventually dies.

Brandt (1958) using fluorescent labelled proteins showed that the initial stage of induction of pinocytosis in amoebae involved adsorption of the inducer on the outer wall. Schumaker (1958) showed that this initial stage is independent of the metabolism of the organism. The adsorption of proteins is a reversible reaction and they can easily be washed off by changing the pH of the solution (Chapman-Andresen and Holtzer 1960). The subsequent stage of pinocytosis in which the inducer and the outer membrane of amoeba are drawn into the cytoplasm is energy dependent (Schumaker 1958).

After ingestion, inducer remains attached to the lining of the pinocytotic vacuoles for some time but it is subsequently released into the lumen. Its fate thereafter is uncertain and seems to vary with the inducer used. There is some evidence that pinocytosis channels and vacuoles develop like food vacuoles. Nachmias and Marshall (1961) deduced pH changes similar to those shown in food vacuoles by Mast (1942). Holter and Marshall (1954) centrifuged amoebae (Pelomyxa carolinensis) in vivo after pinocytosis and showed that the ingested droplets become progressively heavier, presumably as a result of fusion and dehydration.

The possibility has been raised that the membrane of the channels or vacuoles is modified after ingestion. Chapman-Andresen and Holter (1955) showed that glucose, labelled with ^{14}C was not an inducer and did not enter the cytoplasm under normal circumstances. When the same glucose was mixed with a protein, the amoebae pinocytosed vigorously and subsequently showed a diffuse radioactivity on autoradiography and released ^{14}C labelled CO_2 . The authors interpret the results to show that glucose has access to the cytoplasm from the pinocytosis vacuoles. Holter (1961) has discussed the mechanism in its possible relation to therapeutic introduction of non-inducers into a cell.

The electron microscopy of pinocytosis in amoebae might be expected to give more information on the following points:-

1. The mechanism of adsorption.
2. The link between adsorption and cytoplasmic and membrane movement.
3. The fate of the fluid ingested and the mechanism of absorption, the fate of the inducer and of the membrane ingested.
4. Changes in the membrane after absorption possibly relating to its permeability.
5. The relationship between channels and (a) mitochondria, (b) small cytoplasmic vesicles or micropinocytosis droplets.

The adsorption stage has already received some attention. The plasmalemma of normal amoebae consists of two layers (section II) of which the outer takes the form of thin filaments generally considered to consist of acid mucopolysaccharide. Brandt and Pappas (1961) and Nachmias and Marshall (1961) show that proteins and colloidal heavy metal compounds are closely attached to this layer prior to ingestion and the latter authors show that the separation of proteins from the filaments after ingestion depends on their iso-electric point and implies a change in pH of the contents.

The morphology of channels after induction with sodium glutamate was described by Chapman-Andresen and Nilsson (1960) in Amoeba proteus but nothing has appeared on the earliest changes accompanying channel formation. Moreover, most of the studies have so far been made using methacrylate embedding, which is not satisfactory for amoeba (Mercer, 1959). Chapman-Andresen and Nilsson showed that mitochondria and small cytoplasmic vesicles were specifically associated with the channels.

There is no evidence from electron micrographs of the fate of membrane nor of the inducer after ingestion except that Brandt and Pappas (1962) found evidence that colloidal particles entered the contractile vacuoles and were presumably excreted. No definite evidence has been published of qualitative changes in the membranes of pinocytotic vacuoles.

Some additional facts arise from the present study.

1. There is a definite change in cytoplasmic consistency visible on electron micrographs, during early channel formation.

2. Plasma membrane is progressively destroyed after ingestion giving rise in extreme cases to an overloading of the cytoplasm with phospholipid.

3. A new membrane arises from cytoplasmic

constituents to surround and isolate channel fragments.

4. A process of sequestration usually involving mitochondria occurs probably at the peak of channel formation.

MATERIAL AND METHODS

1. The amoebae

The amoebae used for this study were taken from cultures of the following strains maintained at the Carlsberg Laboratory, Physiological Department, Copenhagen:-

Amoeba proteus, Bristol strain, originally established by Sister Monica Taylor and received at the Laboratory from Dr. J.A. Kitching of Bristol in 1953.

Pelomyxa carolinensis (syn, Chaos chaos) also from long established cultures.

Both types of amoeba were cultured in shallow evaporating dishes in a modified Pringsheim's solution (Chapman-Andresen 1958) under conditions of controlled temperature and humidity.

The Amoeba proteus were fed on Tetrahymena geleii which were cultured under axenic conditions, i.e. on a chemically defined medium in the absence of organisms other than chance contaminants. The amoebae received Tetrahymena every other day. Before use in all the experiments they were starved for two complete days.

The Pelomyxa carolinensis were grown under similar conditions but with Stentor sp. as the dominant food organism with added Paramecium and Colpidium.

Experience in the laboratory has shown that this diet gives good results in the centrifugation regime.

Amoebae of both species were handled with fine Pasteur type pipettes, mouth pipettes or Holter's braking pipettes (Holter 1943).

The nomenclature of the two species is discussed in Appendix I.

2. The inducing solutions

Alcian blue. Alcian blue is a basic dye of the copper phthalocyanine series first introduced as a textile dye in 1948 (Haddock). It was quickly adopted as a mucus stain (Steedman 1950) and is popular today alone or in combination with other methods for the detection of acid mucopolysaccharides (Pearse 1960).

Alcian blue has been used to induce pinocytosis and in counting the ingested blue vacuoles (Chapman-Andresen 1963).

In these experiments, alcian blue (Struer's Copenhagen) was prepared as a 1:10,000 solution in glass distilled water. The pH of the water was generally 5.5 - 6.0 and the final solution is acid. In order to produce a suitable degree of pinocytosis the pH was adjusted to 5.8 with N/10 NaOH solution. For one experiment the pH was adjusted to 4.8.

Method for alcian blue solution.

A number of Amoeba proteus usually between 50 and 300 were pipetted with as small a volume of Pringsheim's solution as possible into a small volume of the alcian blue solution, and carefully mixed together. The solution was normally used at room temperature (c. 20°C) but in one experiment both the amoebae and the solutions were left at 4°C in a cold room for one hour before the experiment and the immersion in the dye solution carried out at the same temperature.

The amoebae were allowed to remain immersed for two minutes and then the dye solution was replaced by fresh Pringsheim's solution and the amoebae washed in three changes of the latter. They were left for the desired period (from 5 minutes to six hours) immersed in Pringsheim's solution. In one experiment the amoebae were removed from the alcian blue as quickly as practicable after immersion and fixed immediately. In another the inducer was at pH 4.8 and the amoebae immersed for three minutes before washing.

Sodium chloride solution

To try to maintain correlation with current physiological work (Chapman-Andresen 1963) sodium chloride was used as an inducer as a M/8 solution of sodium chloride (B.D.H. Analar) in M/200 phosphate buffer

modified from Sørensen after Pearse (1960). The buffer was adjusted to pH 6.8.

The amoebae were immersed in a similar way to the alcian blue but left immersed for up to twenty minutes. Specimens were taken from the inducer and fixed at five minutes and ten minutes after initial immersion and the remainder washed and left in fresh Pringsheim's solution for the required period.

Albumin.

Bovine plasma albumin (crystalline, salt free, Armour Laboratory) was used as an unbuffered 0.5% solution in glass distilled water with the final pH adjusted to 4.5 with N/10 HCl.

The same immersion procedure was followed as with sodium chloride. The washing solution was "neutral" Pringsheim's i.e. pH 6.6.

Controls.

A number of series of amoebae were taken straight from the culture medium and fixed to act as controls or normals. The difference in the amount of handling was negligible. Controls were not taken on every occasion as the fine structure of the normal amoeba remains constant.

3. Fixation

Amoebae were fixed in either 1% osmium tetroxide solution (Palade 1952a) or 0.6% potassium permanganate solution (Luft, 1956).

a. Osmium tetroxide solution 40-50 amoebae were added to two ml. of 1% OsO_4 solution made up in the modified Michaelis buffer of Palade (1952) at pH 7.4 at room temperature and gently shaken and left for twenty minutes with occasional slight agitation.

In two experiments normal amoebae were fixed in osmium tetroxide solution at pH 6.0 and 8.0.

b. Potassium permanganate solution similar proportions of amoebae were added to ice cold potassium permanganate solution with the container immersed in crushed ice. The solution was made by mixing equal parts of a 1.2% aqueous KMnO_4 solution and Palade buffer at pH 7.4. (Luft 1956). The whole was left in the refrigerator for twenty minutes.

4. Preparation for electron microscopy

After both forms of fixation the amoebae were washed briefly in distilled water and dehydrated as follows:-

70% ethyl alcohol	five minutes.
90% ethyl alcohol	five minutes.
"absolute alcohol"	three changes of ten to twenty minutes each.

They were then impregnated and embedded in Epon by the method of Luft (1961) with minor modifications to allow for the small size of the material, with propylene

oxide as a clearing agent.

propylene oxide wash briefly

propylene oxide Thirty minutes

propylene oxide plus half the volume of Epon mixture (*) for thirty minutes. Half this mixture was decanted and replaced with fresh Epon and the whole gently mixed. The amoebae were removed from this final mixture of 25% propylene oxide, with as little supernatant as possible and the drop placed on the top of a gelatine capsule filled almost to the brim with Epon mixture. 12 - 20 amoebae were placed in each capsule.

The final Epon mixture (*) was as follows:-

4.2 parts Epon 812

4.4 parts DDSA (dodecenyl succinic anhydride)

1.4 parts methyl nadic anhydride.

to which was added 1.5% DMP 30 (2,4,6, trimethyl amino methyl phenol).

The capsules were placed at 37°C overnight to allow the excess propylene oxide to evaporate and the amoebae to settle to near the bottom of the block, and then moved to 60°C to harden. The hardening process usually took 24 hours.

Comment on the processes used for fixation is included in appendix 3.

5. Sectioning.

The amoebae embedded in Epon blocks were trimmed so that one organism formed the apex of a small pyremidal block. The block was mounted in the chuck of the LKB Ultratome and thin sections cut with a glass knife and floated out on to 20% aqueous alcohol. The thickness of the sections was gauged from the colour in reflected white light. The colours so obtained are referred to in Carasso and Favard (1961) and correlated with the thickness. Sections showing silvery or faintly golden interference colours were selected. The approximate thickness was of the order of 1,000 Å or slightly less. A tentative estimate of section thickness can be made from microg. 10 and is approximately 800 Å.

The sections were picked up on Formvar coated grids (copper, $\frac{1}{8}$ inch diameter, 200 squares).

Subsequently the sections were either stained (below) or examined directly with the electron microscope:-

- i) The Akashi Transscope
- ii) The Philips EM 100b

and photographs of selected areas taken either on 6.5 by 9.0 cm. Ilford Special Lantern Plates at magnifications up to 10,000 times (developed in Agfa Rodinal developer diluted 1:200 for 7 minutes) or on 35 mm Kodak Record

film at magnifications up to 17,000 times (developed in Kodak D.19 B developer for five minutes).

The plates and film were enlarged and printed at a suitable size and a maximum working magnification of up to 120,000 times was obtained.

6. "Staining" Sections were occasionally "stained" in one or other of the following solutions.

a. Karnovsky's alkaline lead solution

(Karnovsky 1961)

b. 10% aqueous phosphotungstic acid (PTA) solution (Latta 1962)

c. alcoholic uranyl acetate solution.

for which the formulae are given in appendix 2. The grids with the sections were floated section downwards on the solutions for thirty minutes to two hours and then washed in distilled water two or three times. They were carefully dried before examination. Only the specimens fixed in osmium tetroxide were stained.

Methods for the centrifugation of *Pelomyxa carolinensis*

Most of the centrifugation techniques were carried out by Mrs. K. Holter.

Specimens of *Pelomyxa carolinensis* selected for their uniformity in size with as near a spherical shape as possible were taken from the cultures mentioned above after starvation for two to four days. They were gently manipulated with a fine pipette and responded by rounding

off and withdrawing their pseudopodia. They were then placed in the chamber of a centrifuging microscope kept at 4°C. The chamber had previously been filled with a gradient consisting of dialysed gum arabic solution with supernatant Pringsheim's solution and a clearcut intervening meniscus (diagram 4).

The chamber was placed in the centrifuging microscope and spun at 5,000 rpm (\approx 2,228 g) for twenty minutes at 4°C with direct visual examination throughout.

During the centrifugation the amoebae settle to the level of the gradient and their cytoplasmic contents stratify according to individual density. At the end of the period the amoebae are removed and fixed in the same way as Amoeba proteus in either osmium tetroxide or potassium permanganate solution. In one case, from which pictures have been used, the amoeba concerned was cut manually into three parts to simulate concurrent biochemical experiments (Holter, Holter and Thompson 1963).

The subsequent treatment of the Pelomyxa carolinensis was exactly similar to that of Amoeba proteus except that the organisms were handled individually and embedded singly. Cutting was done after the block had been rotated so that the amoeba was orientated with its long axis at right angles to the knife. Sections were cut and examined at once to determine the level at which

they had been taken and then collected sequentially to maintain the pattern of the stratifications. Occasional sections were stained in PTA solution.

SECTION 1

THE FINE STRUCTURE OF PELOMYXA
CAROLINENSIS CENTRIFUGED "IN VIVO".

THE FINE STRUCTURE OF PELOMYXA CAROLINENSIS
CENTRIFUGED "IN VIVO"

SECTION I

Heilbrunn (1926; 1929) introduced centrifugation of living Amoeba proteus as a method for the determination of cytoplasmic viscosity. The procedure has been applied to other cell types varying from the sea urchin oocyte (Harvey, 1932) to white blood cells (Bessis and Thiery, 1961).

Centrifugation can provide added information about the cell components in amoebae by stratification according to density. In Amoeba proteus (Mast and Doyle, 1935(b), Singh, 1939) and Pelomyxa carolinensis (Wilber, 1945, Andresen, 1942) it supplements the observations from light microscopy. The increase in density of structures resulting from pinocytosis can be detected (Holter and Marshall, 1954). Centrifugation provides a means of associating biochemical activity with structures too small to be studied individually especially in the localisation of enzymes (Holter and Løvtrup, 1949, Holter and Doyle, 1938, Holter and Lowy, 1959, Quertier and Brachet, 1959) and of DNA (Rabinowitz and Flaut, 1962). Individual components can be removed en masse to determine the effect on the cell (Wilber, 1945) or in order to transfer them to another cell (Daniels and Roth, 1961).

Electron microscopic examination is desirable to establish a base line for such studies and to identify structures whose definition is partly based on their

relative density, such as alpha particles and heavy spherical bodies. A previous electron microscopic study has been made by Daniels and Roth (1961) in Pelomyxa illinoiensis as a control for experiments in irradiation. Amoeba proteus is currently being studied by these methods by Mrs. J.R. Nilsson and is the subject of a brief report by Daniels (1962). This study was initiated in support of other, biochemical, work (Holter, Holter and Thompson, 1963).

1. General observations

Immediately after fixation the polarisation of the centrifuged organism is recognisable by the dark brown osmophilic cap at the centripetal pole and the paler brown centrifugal pole. If allowed to fall freely in an aqueous solution each amoeba rotates so that the pale or heavy end hangs downwards.

2. Electron microscopic observations

The amoeba were orientated at right angles to the plane of sectioning and serial sections cut through their length.

The results are divided into regions as follows according to the predominating structure.

Region 1 - fat

Region 2 - contractile vacuoles

Region 3 - hyaloplasm, cytomembranes

Region 4 - nuclei

Region 5 - mitochondria

Region 6 - a. food vacuoles

b. crystals

c. heavy spherical bodies

} Alpha
particles

The appearance is reconstructed diagrammatically in diagram 3.

There is some degree of overlap especially with the mitochondria which are found in Regions 4, 5 and 6 a and b, and small round vesicles extending from Region 5 throughout the centrifugal pole.

In some specimens the heavy pole proved fragile and the crystals and heavy spherical bodies are missing.

The terms above and below, centrifugally and centripetally and heavier and lighter are used synonymously.

Region 1.

The centripetal pole consists almost entirely of electron dense, amorphous, circular fat droplets. (microg. 1) They range from 2 - 4 μ in diameter with a smooth outline sometimes with a faintly denser peripheral line. The majority lie free in the cytoplasm but occasionally droplets are invested by a loosely applied membrane (microg. 2). In one case there are 2 droplets inside one membrane.

Fat droplets are often closely applied to each

other without losing their shape. Between some, however, there are some membranous sacs $0.5\ \mu$ long, limited by a single irregular membrane.

Region 2.

The cap of fat at the centripetal pole has a fairly sharp lower edge below which the cytoplasm is filled with masses of irregular closed vesicles (see Region 3) and in addition two other structures:-

1. Contractile vacuoles (microg.⁴).

The contractile vacuoles are easily recognised from their appearance in Amoeba proteus and the description by Pappas and Brandt (1958). The wall is smooth with no invagination or outpouchings and has large numbers of satellite vesicles clustered within $1\frac{1}{2}\ \mu$. They range from 1,000 - 2,000 Å diameter. The vesicles are not in continuity with the wall and mitochondria are not found near the vacuole.

The lumen often contains small dense granules of 150 Å diameter which do not occur in the satellite vesicles.

The cytoplasm around the contractile vacuole complex is commonly free of formed elements over wide areas of section.

2. Pinocytotic channels (microg. 5).

Below the level of the contractile vacuoles large "channels" occur, with walls continuous with the plasmalemma and apparently identical with it. The dense limiting membrane is lined with hair like projections similar to those on the plasmalemma (Brandt and Pappas, 1961). The walls of the channels are surrounded to a depth of $1\frac{1}{2} \mu$ with finely granular material which, though it has a sharp edge, is not membrane bound and contains no cytoplasmic structures. The channels have no visible contents. These channels are almost certainly the result of some form of pinocytosis. They are lined with plasma membrane with a filamentous layer and are surrounded by the granular cytoplasm characteristic of pinocytosis channels in Amoeba proteus (section IV). Their origin is not known. There are several possibilities, among them:-

a. that the gum arabic solution (see material and methods) has the properties of an inducer. The high contrast of the filaments of the plasma membrane compared with those of Amoeba proteus prepared by the same methods (section II) suggests there might be some substance adsorbed on to them.

b. that centrifugation induces pinocytosis.

c. that as the rounding off which is an essential preliminary to successful centrifugation, involves sudden loss of surface area, "pinocytosis" may be the mechanism adopted for rapid decrease in surface area by interiorisation of cell membrane.

In any event the presence of channels suggests a method for the introduction into the cell of substances which do not normally induce pinocytosis.

Region 3 (microg. 6).

The remaining material of both Regions 2 and 3, corresponding to the optically empty zone or hyaloplasm (Wilber, 1945) consists of cytomembranes identical to those of normal Amoeba proteus (section II). They decrease progressively in size as the centrifugal pole or heavy end is approached as in P. illinoisensis (Daniels and Roth, 1961).

The limiting membrane of the vesicles is single to low resolution but is irregular and rarely cut at right angles for any distance. The electron density of the contents is similar to the cytoplasm.

After staining by Karnovsky's method or with PTA (microg. 7) granules of 200 Å diameter are seen attached to the outer surface of the membrane. Tangential sections show a whorl-like arrangement like that in Amoeba proteus.

Following PTA staining in this and heavier regions small membranous elements are identifiable by their high affinity for the stain. (microg. 3) They take the form of irregular vesicles with small dense bodies lining their inner surface. The plasmalemma and the filamentous border have a high affinity for PTA and it is suggested that these vesicles are the result of pinocytosis. Whether this is spontaneous pinocytosis as suggested by Roth (1960) and by Daniels and Roth (1961) who found similar structures in centrifuged Pelomyxa illinoensis or the result of secondary pinocytosis from the larger channels found in Region 2 cannot be certain.

Region 4

Region 4 is poorly defined. The nuclei are large and take up most of the sections. The nucleoplasm is similar to that of A. proteus but the nuclear membrane consists of two parallel membranes periodically interrupted by pores. There is no honeycomb structure in Pelomyxa carolinensis.

As well as nuclei there are mitochondria, a few empty vacuoles of 1 - 2 μ diameter and occasional PTA staining vesicles.

Region 5 Mitochondrial zone:

The upper limit of the region blends gradually

with Region 4, the central band consists of close packed mitochondria (microg. 8) with occasional interspersed PTA-staining vesicles and small round vesicles and the lower limit is marked by a decreasing number of mitochondria with an increase in granular background cytoplasm. (microg. 9) Mitochondria are also dispersed into Region 6.

Mitochondria. The mitochondria are very similar to those of A. proteus, usually spherical, perhaps because of centrifugation, with tubular contents (section II). They do not exhibit the regular structure described by Pappas (1959) in normal Pelomyxa.

Small round vesicles

Throughout Regions 5 and 6, as well as the larger cytoplasmic components, there are small vesicular structures in the cytoplasm (microgs. 9,10,11,12). Some of these are intensified with PTA and have been mentioned earlier. The remainder do not stain with PTA and are spherical in shape. Their frequency is least in the lighter mitochondrial region, but increases progressively as the food vacuole region is approached and then decreases in the heaviest parts.

Structurally the small round vesicles consist of a simple, round fairly dense membrane and occasionally especially in the main mitochondrial band, the contents

have a rather denser core.

The diameters of samples of the vesicles from different regions have been measured. They are tabulated in appendix 4 (ii) and graphed in such a way that the distribution of their diameters becomes obvious.

The first region with enough to measure is the main mitochondrial band and the diameters show a range from 800 - 3,500 Å. The arithmetical mean and standard deviation work out as $1,750 \text{ Å} \pm 514 \text{ Å}$ but the histogram (graph 1) shows that most of the vesicles lie between 1,900 and 2,100 Å in diameter. The disparity is the result of the negative skew in the population distribution which may be explained by the method of measurement (appendix 4). Statistical transformation does not eliminate the skew (graphs 2 and 3). For these reasons only the apparent maximum will be considered.

In the more centrifugal mitochondrial region (i.e. between regions 5 and 6a) the population has the appearance shown in graph 5. There are now two distinct maxima. One at 1,500 - 1,700 extending to 1,900 which is apparently slighter smaller than that of the mitochondrial band and a second between 2,700 - 2,900 Å. The numbers are very small and any division between these two populations would be an arbitrary. It does seem

justified to apply statistical tests to the population.

In the food vacuole region (graph 4) there are again two apparent maxima, one between 2,300 Å and 2,500 Å and the other between 3,700 Å and 3,900 Å. Certainly some of the vesicles are the satellite vesicles of food vacuoles but both maxima are at a greater diameter than the corresponding vesicles of Amoeba proteus (section II, appendix 4(1)).

If it is at all possible to synthesise these observations into an overall picture, it seems likely that there are at least two types of small round vesicles on the basis of diameter. The first occupies the mitochondrial region (5) and the second the food vacuole region (6a). The first and smaller group extends into region 6 and possibly decreases somewhat in size as its density increases. There are vesicles of this diameter in the food vacuole region. However, it is not easy to explain the two maxima in the food vacuole region unless yet a third group can be postulated.

The relationship of these small round vesicles of the cytoplasm to the enigmatic alpha particles which lie at the limit of resolution of the light microscope is discussed in section II.

Finally it is possible to compare the satellite vesicles of contractile vacuoles (region 2) with the small

round vesicles of the mitochondrial band. Using a logarithmic transformation of the diameter and Student's t test the populations are significantly different (P is less than 0.05). On the basis of size alone, therefore, the satellite vesicles of contractile vacuoles are not related to the small round vesicles.

Region 6.

Stratification below the mitochondrial region is relatively poor especially where the most centrifugal part of the organism is missing. Region 6, therefore, is divided roughly into three parts by the dominating structure present.

6a Food vacuoles. (microg. 11,12)

The food vacuoles of Pelomyxa carolinensis are still plentiful after two to four days starvation. Most of them contain a little food residue. Their size varies a good deal but is usually in the range 1.5 to 10 μ . The structure is very similar to that of Amoeba proteus (section II). The limiting membrane, which does not stain with PTA, is rarely cut exactly at right angles because of the large diameter and the triple membrane structure characteristic of the plasma membrane has not been demonstrated. The contents consist of debris, presumably food residue and lamellated bodies which are probably the

most constant feature. They are further described and discussed in section II. Bacteria were not seen in the specimens used in this work.

6b. Crystals (microg. 13)

Like the food vacuoles these are very similar or identical to those of Amoeba proteus and the description of the latter (section II) serves for both.

As well as the fully formed vacuoles containing crystal negatives the region has a large number of vacuoles containing an homogeneous substance which is slightly more electron dense than the cytoplasm.

(microg. 13) These may be crystal vacuoles in which the crystal itself lies outside the section thickness, or they may be crystal precursors containing the mother liquor for crystal formation - presumably a nearly saturated solution of triuret possibly with protein admixed.

6c. Heavy spherical bodies (microgs. 13,14).

The heaviest pole of the organisms contains structures similar to some found in Amoeba proteus and interpreted as heavy spherical bodies (section II). They consist of vacuoles ranging from two microns upwards with a strikingly electron dense core. Usually circular and well defined the core is surrounded by an eccentric translucent halo in thin sections which is smaller or

absent in thicker sections. The core is sometimes displaced from the vacuole but the halo does not correspond to the degree or direction of displacement. The appearance is interpreted as the result of sectioning a hard object which has subsequently partially sublimed in the vacuum or the electron beam. The embedding material of thicker sections offers protection against both processes.

Incidental findings:

Golgi material:

Distinctive Golgi membranes have not been seen in Pelomyxa after osmium fixation. However, in one specimen fixed in permanganate and subjected to random sectioning characteristic Golgi membranes were found (microg. 17). The layer is adjacent to mitochondria and a subjective impression at the time was that it may have been the heaviest mitochondrial region i.e. 5 - 6a. Because of the way the specimen had been trimmed no accurate check was possible. It now appears likely from the work of Daniels (1962) that this Golgi region was adjacent to but centripetal to the mitochondrial band i.e. between regions 4 and 5. The interpretation is quite compatible with the micrographs obtained.

Examples of the preservation provided by permanganate are mentioned in appendix 3 and illustrated

by micrographs 15 - 17.

Small dense bodies

The small dense bodies found in relation to the plasma membrane of Amoeba proteus are known to exist in Pelomyxa (Brandt and Pappas, 1961) and are found in centrifuged specimens in the centrifugal portion of the mitochondrial band. Their density must, therefore, be considerably greater than the fat droplets though less than heavy spherical bodies possibly as a result of the presence of a less dense material producing the soap bubble effect mentioned in section II which can also be seen in these examples.

Conclusions:

The general pattern of stratification is similar to that found by other authors. There is disagreement among previous authors regarding the relative positions of the crystals and mitochondria in centrifuged Pelomyxa and Amoeba. Thus Mast and Doyle (1935b), Andresen (1942) and Torch (1959) found that the mitochondria were centrifugal to the crystals whereas Singh (1939) and Wilber (1945) found the crystals occupied the centrifugal or heavier position.

In Pelomyxa illinoisensis Daniels and Roth (1961) found mitochondria and crystals mixed together with the predominance of crystals below the mitochondria and nuclei.

Torch (1959) found that forces as small as 340 g were sufficient to displace mitochondria below the crystals and he is unable to explain the discrepancy. There is no direct evidence from this work but it seems possible that it may be a question of the diet of the organisms prior to use as Andresen (1956) shows a correlation between diet and crystal morphology and also with cytoplasmic viscosity. The gravitational force in this work was 2,228 g. The regional findings are discussed in section II in relation to those of Amoeba proteus.

SECTION II

THE NORMAL ELECTRON MICROSCOPIC
STRUCTURE OF AMOEBA PROTEUS.

THE NORMAL ELECTRON MICROSCOPIC STRUCTUREOF AMOEBA PROTEUSSECTION II

The constituents of Amoeba proteus have been described many times by light microscopy. The most definite contributions are those by Mast (1926) and Mast and Doyle (1935a,b), who succeeded in correlating previous accounts. The difficulties involved were considerable particularly as most previous authors had paid little attention to each others' work and had generally re-named the particles involved or simply described them and rarely given any dimensions. The list of components described here is taken from Mast (1926) with little or no reference to previous authors or precedence. The diagram used by Mast is reproduced as a guide to the description (diagram 1).

Each component is dealt with separately and discussed in the light of relevant material from section I and appendices 4 and 5.

NUCLEUS

Amoeba proteus is usually uninucleate in contrast to the multinucleated Pelomyxa carolinensis (Rice, 1945).

Amoeba proteus was among the first cells whose nuclear membrane was studied by electron microscopy without the use of thin sectioning (Bairati and Lehmann, 1953). The additional facilities provided by thin sectioning have added far more detail (Harris and James, 1952, Greider, Kostir and Frajola, 1956, Schneider and Wohlfarth-Bottermann, 1959, Pappas, 1956, 1959, Mercer, 1959, Roth, Obetz and Daniels, 1960).

The nuclear membrane of the giant amoeba Pelomyxa carolinensis is less complex and more closely resembles that found in other cells (Pappas, 1959).

As a number of authors have shown, the most striking feature of the nucleus of Amoeba proteus is the complex outer membrane of the interphase nucleus, unparalleled in any other cell studied with the electron microscope.

Following permanganate fixation, the membrane appears as two dense lines separated by a translucent space (microg. 19). As far as the number of suitable micrographs is sufficient for measurement each line or membrane has a thickness of 70 - 90 Å with an intervening

space of 150 Å. Occasionally after this form of fixation, the lines are interrupted by spaces of up to 2,000 Å width. The appearance of the spaces does not justify the use of the term pores. The two layers of membrane do not fuse at the edges of the space and though the spaces may be based on genuine structures they are probably artefact.

The nucleoplasm is very poorly preserved after permanganate fixation presumably because the basic proteins are dissolved or remain unfixed (Bradbury and Meek, 1960).

With osmium fixation, a more complex pattern is found (microg. 20 - 24). The cytoplasmic surface is a double membrane of the same dimensions as that found after permanganate fixation, but it is wrinkled and irregular with a variable space between the component layers. Pores have not been recognised in this material when the membrane is sectioned at right angles to its length, though it is probable that this is due to the irregularity of the surface.

From the deep surface of the membrane a series of parallel partitions project into the nucleoplasm. Each consists of a double membrane of approximately 125 Å thickness ending blindly 0.3 μ from the surface. Tangential sections show that the partitions are arranged as a

hexagonal pattern so that a series of cylindrical spaces lie at right angles to the surface. The sections of micrographs 21 - 24 are effectively serial sections through the "cylinders". Where each cylinder reaches the surface the covering double membrane has a target-like appearance formed by a dark circle with a denser central spot. The spaces are $0.25\ \mu$ in diameter and the dimensions of the surface configuration are given in diagram 5. It is possible that the target-like appearance represents a surface pore (Pappas, 1956) but no corresponding discontinuity has been found in the surface membranes.

The nucleoplasm after osmium fixation, contains dense granular peripheral masses or nucleoli (microg. 20). The space between and central to these masses contains the helical structures of Pappas (1956), groups of which are sometimes linked by one end to form bunches. The spirals of the helices are approximately $0.5\ \mu$ in length and, in cross section, $550\ \text{\AA}$ in diameter. They have occasionally been found inside the spaces of the nuclear membrane "honeycomb" (microg. 20).

In the cytoplasm around the nuclei of several amoebae, short straight fibres are found corresponding to those described during mitosis (Roth, Obetz and Daniels, 1960) (microg. 23).

The outer nuclear membrane is sometimes continuous with bleb-like structures projecting into the cytoplasm. Double walled vesicles sometimes lie near the nucleus as if derived from the blebs (microg.24). The blebs are uncommon, but have been noted in other studies after araldite (Mercer,1959) as well as methacrylate embedding (Cohen,1957).

Amoeba proteus has provided a great deal of the material on the interaction between nucleus and cytoplasm. (Brachet,1960) Removal of the nucleus results in loss of motility and cessation of feeding although the effect on the life span is scarcely more than starvation alone (Brachet,1959). Anucleate amoebae respond more vigorously to pinocytosis inducers (Chapman-Androsen and Prescott,1956). In cells in general, the effect of the nucleus is such that material must pass from nucleus to cytoplasm (Mirsky and Osawa,1961) and in order to do so must pass through the nuclear membrane. Ribonucleic acid is synthesised in the nucleus of amoeba and passes into the cytoplasm (Goldstein and Plaut,1955, Prescott,1959). Although pores are common in many types of nuclear membrane (Callan and Tomlin,1950, Afzelius,1955, Watson,1955,1959 and many other authors) the nuclear membrane in metazoan cells does not appear to permit the entrance of large

molecules from the cytoplasm (Feldherr and Feldherr, 1960).

It has been shown, however, that colloidal particles injected directly into the cytoplasm of Pelomyxa carolinensis rapidly enter the nucleus (Feldherr and Marshall, 1962, Feldherr, 1962a) and in doing so are found in the nuclear pores (Feldherr, 1962b). It appears likely that this represents an improvement in technique rather than a qualitative difference from the cells previously studied.

The nuclear membrane of Amoeba proteus contrasts strongly with that of P. carolinensis. The cylinders of the "honeycomb" each have target-like structure at their apex which has been regarded as a pore (Pappas, 1956, Schneider and Wohlfarth-Botterman, 1959) though some authors content themselves with the term annulate structure (Roth, Obetz and Daniels, 1960). No startling difference in the metabolism between Amoeba and Pelomyxa has been reported so that it seems likely that there are pores in the membrane. Preparation of a viable anucleate Pelomyxa is technically extremely difficult. The honeycomb structure disappears during division: it is not visible after prophase and reappears gradually during several hours after the end of cytoplasmic division (Cohen, 1957, Roth, Obetz and Daniels, 1960). Its degree of development

may reflect the maturity of the nucleus and its reappearance is associated with a re-arrangement of the nucleoli to form sheets and masses (Roth et al, 1960). The outer double membrane was present throughout the series of amoebae studied by Roth's group although it lost its continuity.

It is possible that the amoebae fixed in permanganate were in a division stage but as none of those fixed in osmium were without the honeycomb it seems unlikely. In view of the rather destructive nature of permanganate (appendix 3) the honeycomb structure is probably selectively destroyed emphasising the difference between it and the outermost double membrane.

There is no evidence of relationship between the nuclear membrane and cytomembranes, such as exists with endoplasmic reticulum in other cells (Watson, 1955).

Apart from the presence or absence of pores in Amoeba proteus there are other indications that material passes from nucleus to cytoplasm. The surface blebs, (Mercer, 1959, Cohen, 1957) may be a means of transfer but double walled vesicles are rare in the cytoplasm. The presence of helices within the honeycomb structure means that the spaces are accessible to fairly large components of nucleoplasm and if a pore is present may provide a

route into the cytoplasm.

The peripheral masses of the nucleoplasm resemble nucleoli and are generally regarded as such (Pappas, 1956, Mercer, 1959). They stain with haematoxylin but not with Feulgen stain and contain RNA (Heller and Kopac, 1955a, Chalkley, 1936) so the assumption seems justified.

The nuclear helices are difficult to interpret. They are too big for single DNA molecules as their spiral structure might suggest. Pappas (1956) and Roth, Obetz and Daniels (1960) suspect that aggregations of helices may be cross sections of chromosomes.

PLASMALEMMA

Mast (1929), showed that there must be a membrane or plasmalemma limiting the amoeba cytoplasm and the structure of this membrane is now well known from electron microscopic studies. (Greider, Kostir and Frajola, 1956, Cohen, 1957, Pappas, 1959, Mercer, 1959, Schneider and Wohlfarth-Bottermann, 1959, O'Neill and Wolpert, 1961).

The inner layer of plasmalemma proper is a single dense membrane 80 - 100 Å thick which at higher resolution, especially after permanganate fixation can be shown to have a triple layered structure. Micrograph 18 shows this structure after permanganate fixation. The outermost layer is less well defined in the present material. On the outer surface of the plasmalemma proper there is a layer of fine filaments up to 1,000 Å in length packed close together at right angles to the surface. The bases of the hairs are separated from the plasmalemma proper by a translucent space 100 - 200 Å thick. There are sufficient micrographs with this appearance to confirm the findings of others but the outer layer is not visible on all specimens. In the majority there is no layer visible in unstained specimens and a few scraps of filaments after PTA staining. It seems possible that the absence of

the outer layer may be a result of fixation (Szirmai, 1963). Specimens were fixed at different pH values between 6.0 and 8.0 but there was still random preservation of the outer layer. The filamentous layer is never visible after permanganate fixation.

Phosphotungstic acid staining The plasmalemma proper and where visible, the filaments, are intensified by staining the sections with PTA solution and are similar in this respect to the plasmalemma of Polomyxa (Roth, 1960) and of kidney glomerular epithelium (Latta, 1962). Occasionally where the filamentous layer is absent without staining a few remnants near the outer membrane are visible after PTA staining (microg. 84).

The thickness of the plasmalemma proper has been given as 80 - 100 Å (Mercer, 1959, Schneider and Wohlfarth-Bottermann, 1959, O'Neill and Wolpert, 1961) or as 100 - 200 Å (Pappas, 1959, Greider, Kostir and Frajola, 1956). The smaller measurements in these cases, some of which are taken from the figures where not actually stated, are all accompanied by resolution of a triple-layered structure and are therefore more reliable. The resolution of this study is such that while the triple layering is not routinely visible it can be seen in favourable material especially after permanganate fixation.

The triple layering of the plasma membrane is reminiscent of that found in other cell membranes (Robertson, 1959) and combined with studies of phospholipids (Stoekenius, Schulman and Prince, 1960) gives considerable support to the membrane structure advanced by Davson and Danielli (1952). It now seems certain that such membranes consist of a double central layer of polarised phospholipid molecules (see section III). The chemical analysis of Amoeba proteus plasmalemma shows the presence of 32% lipid and 25% protein (Wolpert and O'Neill, 1962). There has been a recent report of an artificially prepared membrane of lipoprotein with electrical properties similar to a cell membrane (Mueller, Rudin, Ti Tien and Wescott, 1962) with a high resistance to direct current and high electrical capacity.

The filaments are an established feature of the cell membrane of Amoeba proteus (Cohen, 1957, Schneider and Wohlfarth-Botterman, 1959, Mercer, 1959, Greider, Kostir and Frajola, 1958) and of Paramecium (Pappas, 1959, Brandt and Pappas, 1961, Roth 1960) and it can only be assumed that the preparatory methods in this material are inadequate to demonstrate them. It is known that the plasmalemma of amoeba contains a metachromatic substance (Bairati and Lehmann, 1953, Spek and Gillissen, 1943); it reacts positively with the periodic acid Schiff technique (Bairati

Lehmann,1953, Brandt, 1958) and stains with alcian blue (section IV, Chapman-Andresen,1963), and must therefore contain a sulphated mucopolysaccharide. The filaments are believed to contain an acid polyglucose (Nachmias and Marshall,1961, Marshall, Schumaker and Brandt,1959, Wolpert and O'Neill, 1962). The attachment of the hairs to the plasmalemma, whatever the translucent substance between the two may be, is strong enough to withstand differential centrifugation procedures but is broken by ultrasonication (Wolpert and O'Neill,1962). The filaments probably play an important part in the initial stages of pinocytosis as a site for the adsorption of inducers (Brandt and Pappas,1961, and section IV). Bennett (1963) has drawn attention to the incidence and importance of extracellular polysaccharides and has proposed the use of the general term "glycocalyx".

The PTA staining of the hairs and plasmalemma is not easily explained. It may simply be a non-specific enhancement of contrast and yet reports of the method show that the plasmalemma of other cells is selectively stained. Latta (1962) suggested that the PTA staining is specific for positively charged groups. There is evidence from the studies of pinocytosis and of membrane potentials (Chapman-Andresen,1963, Bingley, Bell and Jeon, 1962) that the overall change in Amoeba proteus is negative.

Roth, Obetz and Daniels (1960) studied mitosis in a series of eight amoebae and found that the amount of filamentous material on the plasmalemma decreased at certain stages. As the amoeba used in the current study were in interphase, it is felt that such results could have been due to the same poor preservation.

MITOCHONDRIA

Mitochondria were first described at the end of the nineteenth century (references in Benda, 1902) and have since been recorded as an almost ubiquitous cell component.

Cytoplasmic organelles in Amoeba proteus called beta granules, were identified as mitochondria on the basis of their reaction with iron haematoxylin and supravital janus green (Mast, 1926, Mast and Doyle, 1935a). It was not the first use of the term mitochondria in Amoeba (Vonwiller, 1918) but the first correlation with those of other cells. The observations were confirmed in Polomyxa carolinensis by Andresen (1956).

The fine structure of mitochondria has been extensively studied since the first descriptions by Palade (1952) and Sjöstrand (1953). Typically, mitochondria consist of two continuous outer membranes enclosing a space which is incompletely subdivided by transverse, shelf-like cristae (Palade) or internal membranes (Sjöstrand). The exact relationship between cristae and the outer membrane is still not entirely clear (Anderson-Cedergren, 1959).

As a result of biochemical and ultrastructural studies it is possible to make some generalisations about

mitochondrial function.

Mitochondria are the site for enzymes of oxidative phosphorylation and the enzymes of the Krebs cycle are exclusively localised in their structures (Schneider and Hogeboom, 1956).

A large number of other oxidative enzymes are also present (for reference see Novikoff, 1961a). The localisation has been confirmed, especially in the case of succinic dehydrogenase by histochemistry, both in the light (Nachlas, Walker and Seligman, 1958) and electron microscopes (Barnett and Palade, 1958) and in Amoeba proteus, by centrifuging the mitochondria to one pole and analysing biochemically (Holter, 1955).

The respiratory enzymes are localised on the mitochondrial membranes (Barnett and Palade, 1958) probably in regular sequences (Green, 1958). Approximately 20% of the membranes may be enzyme protein (Lehninger, 1959). The activity of the mitochondria is transmitted by means of adenosine triphosphate and 90% of a cells ATP production is by the mitochondria (Lehninger, 1959, Novikoff, 1961a).

The matrix contains proteins, nucleotides, electrolytes and enzyme substrates and probably the fatty acid oxidation enzymes (Novikoff, 1961a, Carasso and Favard, 1961).

Mitochondria may be capable of independent protein synthesis (Bates, Craddock and Simpson, 1958, Bates, Kalf and Simpson, 1959). They are believed to contain ribonucleic acid (Schneider and Hogeboom, 1951) but such results may be partly due to contamination during preparation (Novikoff, 1961a).

In common with those of other protozoa (Sedar and Porter, 1955, Sedar and Rudzinska, 1956) the mitochondria of Amoeba proteus and Pelomyxa carolinensis have an internal system of tubules instead of cristae. (Pappas, 1959, Mercer, 1959, Schneider and Wohlfarth-Bottermann, 1959, Cohen, 1957, Greider, Kostir and Frajola, 1958). A similar structure occurs in steroid secreting cells (Belt and Pease, 1956) and some plant tissues (Lance, 1958).

In the present study typical "tubular" mitochondria are found throughout the cytoplasm and no other type has been seen (microg. 25).

They are relatively less frequent near the plasma membrane and are specifically associated with the contractile vacuole as a layer of variable depth outside the microvesicular layer (Pappas and Brandt, 1958) (microgs. 26, 29, 30, 31).

Each mitochondrion is limited by two approximately parallel membranes which completely separate the matrix from the cytoplasm. The matrix contains a variable number of circular or elongated profiles of tubules in section, each of which is bounded by a single membrane often continuous with the inner lining membrane (about 4 times in each section). The tubules represent villus-like invaginations of the inner lining membrane into the matrix.

The mitochondrial membranes can therefore be regarded as separating three "phases"

1. the cytoplasm
2. the space between the limiting membranes continuous with the intratubular space
3. the mitochondrial matrix proper or intertubular space.

The electron density of each compartment is similar but the matrix contains poorly defined fibrils.

The dimensions of the mitochondria are remarkably variable. In section they may be round or slightly elongated with a (transverse) diameter of 1 - 2.5 μ and a length up to a maximum of 5 or 6 μ . There is no correlation between size and position (e.g. with regard to contractile vacuole). The tubule dimensions are constant in any one section but vary rather widely

over a sample. Measurements were made of tubules obviously cut at right angles and in 46 mitochondria measured, 29 had a tubule diameter between 800 and 1,000 Å, 11 between 600 and 800 Å and 6 between 400 and 600 Å.

Following section staining by Karnovsky's method, small, moderately dense 200 Å granules are distributed through the matrix (microg. 25). They are similar in appearance to those on the outer surface of cytomembranes but are not attached to the tubular membranes.

After permanganate fixation, the mitochondria are grossly modified and vary from specimen to specimen. The commonest picture shows loss of tubules with no increase in size to indicate swelling (microg.45). In many cases there are two outer membranes but they are separated by tubule-like structures. The appearance in microg. 44 suggests these may be the beginning of myeloid figures. As these are so characteristic of phospholipids of which mitochondria contain a great deal they may well be due to partial extraction of protein and collapse of formed structure into the most stable form (appendix 3).

Quantitative studies: It is possible to estimate the proportion of the volume of a mitochondrion occupied by

its tubules by employing the method recently published by Loud (1962). The relative volume (i.e. volume of tubules, volume of mitochondrion) is almost numerically equal to the relative area of each on the print (i.e. area covered by tubules/total area covered by mitochondrion). The relative areas can be estimated by superimposing a grid of equidistant parallel lines on the print and measuring the ratio of length of lines intersected by tubules to total length of lines intersecting whole mitochondrion. The ratio can be read off as the relative volume occupied by the tubules.

The results of applying this test to eight mitochondria is given in appendix 5. The volume ratio varied from about 13% to 30%.

It has been suggested elsewhere (Palade, 1953, Lever, 1955, Belt and Pease, 1956) that "tubular" mitochondria offer a greater surface area for enzyme activity than those with cristae. A theoretical consideration of idealised forms has been made (appendix 5) and it is found that the ratio:-

$$\frac{\text{Surface area of tubules, relative to mitochondrion}}{\text{Volume of tubules relative to mitochondrion}}$$

is mathematically extremely simple in idealised forms. It is twice as great in cristaeform mitochondria as in tubular mitochondria when the diameter of the mitochondria

and the thickness of tubules and cristae are the same. Some implications and possible applications are discussed below.

The relative volumes occupied by cristae of mitochondria of some metazoan tissues are also estimated using Loud's method (appendix 5). The magnitude can be seen to be of the same order as the mitochondria of Amoeba proteus.

The findings in this study confirm those made previously and already mentioned in Amoeba and Pelomyxa. They show that individual mitochondria are large and combined with quantitative studies that as might be expected they have a very large surface area between the matrix and the intratubular space (appendix 5).

The damage which occurs in sequestrations and after the enforced absence of protoplasmic streaming in intense pinocytosis (section IV) suggest that these large complex mitochondria require frequent renewal of their surrounding medium to retain their structural characteristics. Palade (1956) suggests that all mitochondria have this requirement but it seems possible that individual size and complexity is facilitated by the more rapid flow. Since the contractile vacuole mitochondria are identical with the remainder they probably undergo repeated inter-

changes with the general pool.

It is of interest from the figures obtained in appendix 5 to have calculated the total surface area of the tubules, the area into which the total oxidative enzyme capacity consisting of Green's units (1958) must be incorporated. A tentative approximation of this is 0.35 mm^2 per amoeba.

Some attempts are now being made to correlate mitochondrial structure and volume with enzyme activity. Mattiesson and Birch-Anderson (1962) have shown that mitochondrial structure can be matched with the enzyme pattern of terminal respiration in different taxonomic groups of invertebrates. There is a positive correlation between the capacity of the cytochrome system and the number of mitochondria as well as the number of cristae. A.D. Hally (personal communication) has shown a correlation between enzyme activity and the volume of a cell occupied by mitochondria.

In a very few examples of metazoan tissues (appendix 5) the relative volume of the mitochondria occupied by cristae is of the same order and range as the relative volume occupied by tubules in Amoeba proteus. A combination of observations and theory produces relative surface areas in both groups and it is clear from this very small selection that the relative surface area

for amoebae is far smaller than that in the more active metazoan tissues.

The volume of the whole cell taken up by mitochondria in amoebae is far less than in say gastric parietal cell where it is of the order of 30% (Hally, personal communication). The selective arrangement of mitochondria around the contractile vacuole leads to a local but unmeasurable increase in the volume occupied.

It appears that in amoeba, available mitochondrial material is organised in large units with a relatively small area of enzyme surface and that this arrangement may require highly active streaming cytoplasm.

CYTOPLASMIC MEMBRANE SYSTEMS

Vacuolation is a feature of the cytoplasm of the amoebae. Andresen (1956) describes a number of vacuoles in detail in Pelomyxa and various forms have been described in Amoeba proteus by electron microscopy (Mercer, 1959, Pappas, 1959). The excellent preservation provided by epoxy resin embedding enables a more general view to be taken and, in combination with centrifugation studies make it possible to classify the vacuoles accurately.

1. The cytomembranes (microgs. 27, 68, 82). Loose irregular membrane bound sacs which do not communicate with each other are found throughout the cytoplasm. The outer membranes have a thickness of about 100 Å but because of their irregularity are rarely cut perpendicularly for any distance. Under the same conditions of preparation they are far less dense than the plasma membrane and do not stain with PTA (microg. 50).

The outer surface of the sacs is studded with small round particles of about 150 Å diameter arranged singly or in groups often in the form of a whorl. The granules are more abundant after heavy metal staining particularly with lead by Karnovsky's method (microgs. 25, 28 and 50).

The membranous sacs have no visible contents and in normal organisms bear no relationship to other cytoplasmic constituents. Their possible relationship to the new membrane formed after intense induced pinocytosis is discussed in section IV (microg.82).

2. Large vacuoles Spherical vacuoles with a diameter of from 1 to 4 μ have a smooth dense membranous wall similar to the plasma membrane, which is often studded with small dense bodies on the cytoplasmic surface. The vacuoles usually have no contents and bear no relationship to other structures. The walls, unlike plasma membrane, do not stain with PTA. Sometimes the small dense bodies are found inside the vacuoles forming a shell (microg.32).

3. Small round vesicles (alpha particles) (microg. 39)

Among the membranous elements of the cytoplasm, small spherical bodies can be recognised. They occur in small numbers at random and are within the range of 0.1 - 0.3 μ in diameter. The numbers are too small to be more precise. The walls are smooth, PTA negative, with no associated dense bodies and relatively thick, appearing in section as a dense line. The contents are usually the same density as the cytoplasm but rarely there is a faintly denser core.

Morphologically the vesicles look the same as

the satellite vesicles of the food vacuoles (microgs.36, 37,46). As the latter are more frequent they can be measured more accurately (appendix 4). In a series of 14, the mean diameter was $1,830 \text{ \AA} \pm \text{s.d. } 1,070 \text{ \AA}$ with a complete range of $0.1 - 0.45 \mu$.

It is worth considering the sizes of other vesicles. The contractile vacuole satellites (mean of 25; $1,020 \text{ \AA} \pm \text{s.d. } 960 \text{ \AA}$; range $400 - 2,500 \text{ \AA}$) are significantly smaller ($P = \text{less than } 0.01$; P calculated from Student's t test from logarithmic distribution (appendix 4)) but those of the Gelgi zone (series of 8, mean $1,650 \text{ \AA} \pm \text{s.d. } 450 \text{ \AA}$) are not significantly different to the food vacuole satellite vesicles ($P = 0.75$). On the basis of size alone the satellite vesicles of the food vacuoles and contractile vacuoles are not the same although this does not preclude a developmental relationship. Further observations on size are available from the centrifuged studies (section I).

Discussion

The cytomembranes are described by Pappas (1959) who suggested that they were analogous with the endoplasmic reticulum of other cells. There appear to be more granules in the present material after staining than in that of Pappas in unstained methacrylate sections.

The granular endoplasmic reticulum, first

described in whole cultured cells under the electron microscope (Porter, Claude and Fullam, 1945, Porter and Thomson, 1947) is the ultrastructural representative of the ergastoplasm (Haguenau, 1958) and accompanies cytoplasmic basophilia. (Porter, 1954, 1961) Basophilic cytoplasm contains ribonucleic acid (Brachet, 1950) and studies by Palade and Siekevitz (1956a,b) have shown that the granular element of the ER contains the RNA. The microsomes of differential centrifugation which are rich in phospholipids and RNA (Claude, 1941) represent fragmented endoplasmic reticulum (Palade and Siekevitz, 1956). Studies on radioactive amino acid uptake show that the microsomes are the principal if not the only site of protein elaboration in the cytoplasm (Hultin, 1950). The endoplasmic reticulum is the cytoplasmic protein factory. The ER and the microsomes have been shown to contain large numbers of enzymes (Porter, 1961) and recent work shows that these may be associated with the membranes or with the intermembranous space (Ernster, Siekevitz and Palade, 1962). Important functions are now ascribed to a non-granular ER notably in relation to carbohydrate metabolism (Fawcett, 1955, Karrer, 1960a,b), steroid secretion (Christenson and Fawcett, 1961) and lipid metabolism (Palay, 1958).

In Amoeba proteus, although the nucleus is active

in protein synthesis and exerts a profound effect on the cytoplasmic RNA and protein synthesis, protein is definitely synthesised in the cytoplasm (Mazia and Prescott, 1955). It is known that most of the RNA of the cytoplasm in Amoeba is found in microsome-like particles (Brachet, 1960). The cytomembranes are considered to be the counterpart of the protein synthesising endoplasmic reticulum. The analogy rests on the association of small granules and membranes and the absence of an acceptable alternative. Morphological differences from the ER might be due to the constant cytoplasmic streaming which may make the presence of interconnected flattened sacs impossible. It is worth noting here that other Sarcodina have a typical ER (Pappas, 1959). The greatest elaboration of the granular endoplasmic reticulum occurs in exocrine secretory cells with a high protein output (Porter, 1961). Amoeba proteus produces protein for its own maintenance only and for the synthesis of new material in preparation for division. The paucity of the cytomembranes and the sparseness of the attached granules is probably related to the diffuse basophilia of the cytoplasm of Amoeba proteus (Brachet, 1950). The accentuation and even the increase in number of granules with heavy metal staining might indicate a lower osmophilia than in other cells. Possibly there is a lower

protein content since proteins are a major factor in osmophilia of non-lipid structures (Bahr,1954).

There is no association between the cytomembranes and the nuclear membrane or the Golgi apparatus such as occurs in some mammalian cells (Watson,1955,Palay,1958).

The cytomembranes of Pelomyxa carolinensis on centrifugation occupy the optically empty zone or hyaloplasm and their identification as the RNA containing membranes of the cytoplasm goes some way to explain the shortening of the life span that occurs when the hyaloplasm of Pelomyxa is removed (Wilber,1945). It is of interest that the removal of the mitochondria in the same specimens did not decrease the life span further. The cytomembranes are a greater limiting factor in this respect. They may also be the site of those enzymes found in the light half (Holter and Løvtrup,1949).

Large cytoplasmic vacuoles with walls similar to the plasma membrane are described or illustrated in other studies (Greider,Kostir and Frajola,1956, Schneider and Wohlfarth-Botterman, 1959, Mercer, 1959, Roth,1960). Some authors are able to show a triple membrane structure in the vacuole wall similar to the plasmalemma. The origin of the vacuoles is not clear. Roth (1960) is of the opinion that many represent spontaneous pinocytosis vacuoles. Spontaneous pinocytosis has received little attention but

Wolpert and O'Neill (1962) think it may be the mechanism by which slow membrane turnover occurs. They identified the walls of the cytoplasmic vesicles as plasmalemma by their reaction with labelled antigen prepared against isolated plasmalemma.

There is no evidence of large numbers of empty cytoplasmic vacuoles in the centrifuged Pelomyxa except for a few large ones in the nuclear region. The vacuoles with homogeneous contents found in the crystal zone have been taken to be "non-crystal bearing" crystal vacuoles or potential crystal vacuoles, which may contain a saturated solution of crystal material. It is possible that some of the vacuoles of Amoeba proteus are of the same type.

Although the walls of the cytoplasmic vacuoles resemble the plasma membrane they do not stain with PTA. The walls of pinocytosis vacuoles may change in their physical and chemical properties after ingestion (Chapman-Andresen and Holter, 1955, Brandt and Pappas, 1962).

The small round vesicles found in Amoeba proteus are morphologically comparable to those in the heavier layers of Pelomyxa. Similar particles have been found in the centrifuged Pelomyxa illinoisensis (Daniels and Roth, 1961). The size and distribution of these particles suggest that they are the alpha particles of light

microscopy so that only the larger ones would have been visible in the earlier studies. Alpha particles have been seen in nearly all light microscopic studies but missed in most electron microscopic studies so far. No histochemical information is available (Pappas, 1954, Heller and Kopac, 1955a), possibly because they are at the limit of resolution for light microscopy. Cohen (1957) identified a structure as the alpha particle but his illustrations show something far bigger than their established size. It seems likely that the particles escaped identification because of poor preservation in methacrylate and because they are regarded as part of the general membrane system.

The function of the alpha particles is obscure and measurements made in centrifuged Polomyxa suggest a heterogeneous population on the basis of diameter. The smaller group sediment with the mitochondria. The larger vesicles are concentrated in the food vacuole region (6a) suggesting that though bigger they may have a higher density. Their distribution may not however, be based entirely on density as like the satellite vesicles of the contractile vacuoles, their association with the food vacuoles might be a stronger influence than the centrifugal field. If they had recently been formed from the food vacuoles, as it is generally assumed (Roth, 1960) their density would probably

be much the same.

It has been informally suggested that they may be fusing with rather than separating from the main vacuole (Holter, personal communication). The tenuous evidence from the Golgi apparatus is of interest in this respect (see page 90). Brandt and Pappas (1962) were able to show ingested colloidal material in the satellite vesicles of pinocytosis vacuoles and in the satellite vesicles of the contractile vacuoles. The possibility of direct transfer is discussed on page 73.

THE CONTRACTILE VACUOLE

Fresh water organisms such as Amoeba proteus and Polomyxa carolinensis which ingest fluid during feeding by phagocytosis and possibly by spontaneous pinocytosis and in which the outer medium has a lower osmotic pressure than the cytoplasm must have a means of eliminating excess water. The visible means is provided by the contractile vacuole (Kitching, 1956a).

A. proteus has a fairly constant site at which one contractile vacuole forms but P. carolinensis has numerous vacuoles (Rice, 1945).

The cycle of activity is well known (Mast, 1938, Andresen, 1942, Kitching, 1956). Initiation of a vacuole, usually at the vestige of the old one (Mast, 1938) but sometimes near a newly formed food vacuole (Andresen, 1956) is followed by progressive increase in size or diastole during which time the vacuole lies free in the endoplasm and finally rupture or systole after entering the ectoplasm (Kitching, 1956a).

The contractile vacuole has a coating of beta granules or mitochondria (Vonwiller, 1918, Mast, 1938) and a surrounding layer of yellowish hyaline cytoplasm (Andresen, 1956). The fine structure has been described by Pappas and Brandt (1958) and this work confirms their

observations.

Observations (microgs. 26,29,30,31)

The contractile vacuole is irregular with a single moderately dense outer membrane and basic contents of the same electron density as the cytoplasm.

Occasionally the contents include:-

1. very small particles of 100 - 200 Å diameter of moderate density and grouped in clusters.
2. small dense bodies of the type attached to the plasma membrane of 1,000 Å diameter.

The cytoplasm surrounding the vacuoles contains

1. numerous vesicles either round or elongated, congregated within about 1 μ of the wall and more or less evenly distributed through this depth. The vesicles range from 400 - 2,000 Å in diameter (mean 1,070 Å ± s.d. 660) and those nearest the wall of the vacuole especially the elongated ones maybe in continuity with the wall. The background cytoplasm in the microvesicular layer is patchily granular.

2. at a greater distance - 2 or 3 μ from the wall there is a layer of mitochondria, one or two deep. They always lie outside the vesicular layer and are sometimes packed so closely that they touch each other. They do not differ from the remaining mitochondria of the cell. The thickness of the mitochondrial layer is very

variable and occasionally quite sparse. The number is not correlated with the number of small vesicles. No attempt was made to follow the development of the contractile vacuole.

Discussion

Kitching (1956a) ascribes the mechanism of diastole to:

- a. osmosis
- b. phase separation.
- c. active transport or secretion of water with or without modification of the fluid by active transport across the membrane.

Pappas and Brandt (1958) propose that the small vesicles arise in the surrounding cytoplasm and coalesce with the vacuole and each other. In forming they provide a large surface area across which water and/or ion transport can occur. They did not suggest the mechanism for vesicle formation but it would presumably come into the category of phase separation, after the manner of coacervation in colloidal suspensions described by Bungenberg de Jong and Bonner (1935). Such a process has not been established electron microscopically but the spontaneous formation of a membrane by "surface precipitation" around isolated cytoplasm has been postulated by Heilbrunn, Ashton, Feldherr and Wilson (1953).

Brandt and Pappas (1962) found evidence of pinocytosed material in Pelomyxa carolinensis in both satellite vesicles of pinocytosis droplets and satellite vesicles near the contractile vacuole. They suggest therefore, a circulation between ingested droplet and contractile vacuole. The view is an attractive one especially as Andresen (1956) could see new contractile vacuoles forming around food vacuoles. There are objections to such an unmodified mechanism however.

Firstly on purely morphological grounds, the size of vesicles around food vacuoles (average $1,830 \text{ \AA} \pm \text{s.d. } 1,070 \text{ \AA}$) is significantly greater than those around the contractile vacuole (average = $1,020 \text{ \AA} \pm \text{s.d. } 960 \text{ \AA}$) (P less than 0.01, calculated from logarithmic distributions) (appendix 4(i)). This might be explained by shrinkage "en route". Secondly micropinocytosis without a net change in surface area leads to a gross loss in volume. If a droplet 10μ in diameter gives rise simply by "pinching off" to a vesicle 0.4μ in diameter without gain in total surface area or available membrane, the loss in volume is equal to 37.5 times the volume of the small vesicle (appendix 6). Moreover of course the available membrane of the larger droplet is sufficient to cover only a very small number of vesicles. In a similar way, the fusion of small droplets must either

be accompanied by a big increase in volume or the accumulation or destruction of membrane. There is no evidence for accumulation.

There is evidence that amoebae are not capable of rapid membrane turnover (Chapman-Andresen and Dick, 1961a, Wolpert and O'Neill, 1962, and section IV) but it is just conceivable that there is an equilibrium between membrane components and cytoplasmic lipoproteins so that membrane can form spontaneously by "precipitation" when required and disperse when in excess.

If the proposed circulation is accepted it would provide a mechanism for the membrane circulation postulated by Wolpert and O'Neill (1962) but without overall gain or loss of membrane it must be accompanied by simultaneous transfer of fluid to and from the cytoplasm.

The presence of mitochondria is said to be unnecessary for normal function of the contractile vacuole (Wilber, 1945) but at the same time the vacuolar mechanism is inhibited by respiratory poisons (Kitching, 1936). Contrary to the view that only water is excreted (Kitching, 1936, 1956a) it has been shown that some sodium passes out (Chapman-Andresen and Dick, 1961b). It is highly unlikely that the animal can afford to lose sodium in great quantity and the mitochondria probably serve the

same function as they do in the ion exchange systems of kidney (Rhodin, 1954) and gastric parietal cell (Hally, 1959). Simply looking at static micrographs of the contractile vacuole can add little more to the understanding of its function.

The mechanism of inhibition during pinocytosis is unknown (Chapman-Andresen, 1963).

FOOD VACUOLES

Both species of amoeba ingest live food, usually ciliate protozoa, by phagocytosis (Kudo, 1946, Kitching, 1956b). Some external medium is taken in simultaneously. The course of development of food vacuoles has been followed by Mast (1942) and by Roth (1960). After ingestion there is a rapid decrease in size which is apparently osmotic (Kitching, 1956b) and a fall in pH either as a result of the respiration of the food organism (Mast, 1942) or secretion of acid. After the organism dies the pH rises and the volume increases once more as digestion begins. There is finally a slow decrease in volume, presumably as digestion products are removed.

All the amoebae of this study were starved for two days and the food vacuoles were all in the process of decrease in size. No attempt was made to follow their earlier development.

The food vacuoles are approximately circular (microg 35, 36, 37, 46). The outer membrane is comparable to the plasma membrane in density and thickness with small dense bodies attached to its outer or cytoplasmic surface. It does not however stain with phosphotungstic acid and as high

resolution studies are not available it is not possible to confirm a triple membrane structure.

The contents of the food vacuoles consist of:-

1. debris
2. lamellated masses
3. bacteria

all of which lie in a background of faintly granular flocculated material presumably representing precipitated fluid contents, with a granule free peripheral space. The food debris is unrecognisable as the remains of the food organism and may well include other ingested debris. The membrane fragments usually stain heavily with PTA. The lamellated masses occur in roughly spherical lumps and masses often with a central space and inter-connected with lamellated strands. Individual masses have no limiting membrane. A higher magnification of the lamellated masses shows them to consist of approximately parallel osmophilic lines 40 - 50 Å wide, separated by translucent spaces of variable width (microg.38). The relationship of such masses to phospholipids is discussed in section III.

The bacteria of the food vacuoles are morphologically identical with those of the bacterial complexes. They are not specific contents of food

vacuoles as suggested by Brandt and Pappas (1962).

The origin and structure is considered in section III.

Discussion

The outer membranes of food vacuoles is derived from the plasma membrane during phagocytosis and in the early stages is identical with the plasmalemma and stains with PTA (Roth, 1960). In this material the membrane is PTA negative, has no filamentous lining and has not been resolved as a triple layered structure. Physiological or structural differences from plasmalemma like those suggested by Kitching (1956b) and by Chapman-Andresen and Holter (1958) for pinocytosis vacuoles have not therefore been excluded.

The bacteria are discussed more fully in section III. It is assumed they are ingested during phagocytosis but their apparently healthy state has not been explained.

It has been well established in electron microscopy that lamellation such as that found in food vacuoles is characteristic of phospholipids with or without protein (Stoekenius, Schulman and Prince, 1960, Mercer, 1961, Finean, 1961). Cell membranes of all types consist of three layers - the "unit membrane" structure (Robertson, 1959) with a total thickness of about 75 Å of which the two outer thirds are osmophilic and the inner third relatively electron translucent. Artificial

preparations of pure or crude extracts of phospholipids (Revel, Ito and Fawcett, 1958, Stoekenius, 1959, Stoekenius et al, 1960, Mercer, 1961) have the same structure but with a repeating width of 40 Å. Addition of protein to an artificial preparation leads to an increased thickness (Revel et al, 1958). The difference in thickness of naturally occurring lamellae has been employed to distinguish sites of phospholipids from lipoprotein depositions (Thoenes, 1962).

In the present case the thickness of individual bands is approximately 40 Å and suggests they may represent protein free phosphatides (section III). Phosphate incorporation during phagocytosis in leucocytes may show lipid metabolism to be the limiting factor in phagocytosis (Karnovsky and Wallach, 1961). There is ample source for phospholipids in the structural lipoproteins of food organisms and its presence in food vacuoles suggests the protein fraction is more easily dealt with.

The satellite vesicles of food vacuoles, which form one of the populations of small vesicles in centrifuged Pelomyxa may be part of the group known as alpha particles (Mast and Doyle, 1935a, Andresen, 1942).

SMALL DENSE BODIES

A number of authors have found small dense bodies in either Amoeba proteus and Pelomyxa carolinensis. In some cases they are merely described (Cohen, 1957) but others have dismissed them as artefact (Brandt and Pappas, 1961). Mercer (1959) suggests they might be similar to the volutin of bacteria and Greider, Kostir and Frajola (1959) compare them to "neutral red granules" and believe their electron density to be due to a lipid but at the same time claim they are present after formalin fixation.

The plasma membrane is regularly studded on its cytoplasmic surface with small very dense bodies which are circular except where they are attached to the membrane. The association is so close that the membrane is invisible at the point of attachment. The bodies have a very high electron density, comparable with that of the heavy spherical bodies. They are approximately 1,000 Å in diameter and in many cases are slightly lobulated with an internal structure of small bubbles of 100 - 200 Å diameter. The bubbles may be the result of damage from heating in the beam (Cohen, 1957, Mercer, 1959) (micrographs. 33, 70, 78, 80).

The dense bodies are most commonly and easily seen on the plasma membrane. They are however associated

with other cytoplasmic vesicles of uncertain origin and, in experimental organisms with pinocytosis channels and droplets. In addition to these structures which might all have been derived from the plasma membrane, the bodies occur on the outer surface of fat droplets (microg.39). The dense bodies have not been seen in the nucleus.

Small particles of dense debris, smaller than the above and with no internal structure are commonly found in the cytoplasm, and sometimes in food vacuoles and contractile vacuoles. Experimental organisms show that such debris tends to accumulate at the edge of granular cytoplasm associated with pinocytosis channels (microg. 71).

Small dense bodies are also found inside cytoplasmic vacuoles. Micrograph 32 shows a vacuole which contains a shell of dense material consisting of small dense bodies linked by threads. In such a case the outer membrane is free of dense bodies and the appearance suggests that material initially attached to the outer membrane has become transferred to the lumen by some unidentified process.

The dense bodies associated with the plasma membrane sometimes disappear from the section leaving a small punched out translucent space. There are always some neighbouring bodies present. In sections stained with

PTA (pH 1-2) the dense bodies disappear leaving similar spaces (microg. 79). They appear to be soluble in dilute acids. After potassium permanganate fixation the dense bodies are present with the same density and structure as in osmium fixed tissue (microg. 34).

Discussion

The small dense bodies of the plasma membrane constitute a definite entity which is distinct from the occasional debris of the cytoplasm. They are soluble in dilute acids and present after permanganate as well as formalin (Greider, Kostir and Frajola, 1959). It seems most likely that their electron density is inherent and comparable to that of the heavy spherical bodies. Their position after centrifugation shows them to be heavier than fat but lighter than heavy spherical bodies possibly because they have a lighter substance in the bubble-like internal structure. The possible relationship between heavy spherical bodies and volutin is commented on later and there is no reason to contradict Mercer's suggestion (1959) of a similarity between dense bodies and volutin. Volutin is an inorganic polymetaphosphate (Glauert and Brieger, 1955, Baker, 1958).

The selective association with membranes is not absolute as it can be broken down by centrifugation but it suggests that fat droplets might have an outer membrane

to which the dense bodies are attached. Some fat droplets in Pelomyxa have an outer membrane (section I)

The nature of the dense bodies is still unknown but they seem too well defined with a regular distribution to be dismissed as artefact.

HEAVY SPHERICAL BODIES

A class of large granule is found in both Amoeba proteus and Pelomyxa carolinensis as well as related forms. It has received a variety of names of which a comprehensive list is provided by Mast and Doyle (1935a) who refer to this granule as a "refractive body". Andresen (1956), pointing out that the granule stains with neutral red in Amoeba proteus but not in Pelomyxa calls them the heavy spherical bodies. Most German authors use the term "Glanzkörper" after Groeff (1874). The granules accumulate in the most centrifugal pole in both species and this constitutes their most characteristic feature. Some chemical findings have been published. The heavy spherical bodies are metachromatic (Heller and Kopac, 1955a) and contain mineral salts (Heller and Kopac, 1955b). They are said to have an outer shell containing fatty acids (Mast and Doyle, 1935a) and proteins but are periodic acid-Schiff negative (Pappas, 1954).

If the heaviest pole of an amoeba is removed after centrifugation the heavy spherical bodies reappear in the recovering amoebae. (Mast and Doyle, 1935b)

Observations (microg. 39)

A structure which could not be equated with any of those previously described consists of a circular membrane 2 - 4 μ in diameter enclosing material similar in density to the background cytoplasm with a central core of very dense material. The core is more electron dense than the fat droplets and comparable to the small dense bodies. It is not complete in all the examples but is better preserved in thicker sections. In general, it is partially surrounded by an eccentric translucent halo similar in density to the crystal negatives, which sometimes has a dark smudge at one edge. Although the core retains its smooth outline at the site of the halo there is some loss of material as if by sublimation in the beam or in the vacuum.

As they do not correspond to a recognised structure in the electron microscope it was thought that these bodies might be either alpha particles or heavy spherical bodies. The former have been tentatively identified by Cohen (1957) but for reasons already given his description is not acceptable. The structures described here are too big and too scarce to be alpha particles which are about 0.25 μ in diameter (Mast and Doyle, 1935a). Examination of centrifuged Pelomyxa carolinensis (section I) proves that identical structures are found in the most centrifugal

part of the preparation - the site occupied by the heavy spherical bodies (Andresen, 1942, Wilber, 1945).

Discussion

Although neutral red staining in vivo gives evidence of dissimilarity between the heavy spherical bodies of Amoeba proteus and Pelomyxa (Andresen, 1956, Pappas, 1954) the electron micrographs leave little doubt that the ultrastructure is very similar in the two species.

The heavy end of centrifuged Pelomyxa is not osmiophilic and the high electron density therefore indicates the presence of elements of a high atomic number (Bahr, 1954) probably minerals, confirming microincineration and histochemical studies (Heller and Kopac, 1955b, 1956). In combination with metachromasia (Heller and Kopac, 1955a) this recalls the polymetaphosphates or volutin of bacteria and yeasts (Baker, 1946, Wiame, 1947), which is equally electron dense (Glauert and Drieger, 1955, Glauert, 1962). The volutin granules of bacteria disappear from thin sections in a similar way to heavy spherical bodies (Vanderwinkel and Murray, 1962). Mercer (1959) suggests that small dense bodies might be volutin and it can only be said that their electron density is very similar in this study.

The ultra-structure of the heavy spherical bodies is difficult to correlate with the histochemistry. The

outer shell presumably corresponds to the relatively translucent material within the limiting membrane but there is no support for the demonstration of lipid or protein.

THE GOLGI APPARATUS

Since the first description of the Golgi apparatus in thin sections of epididymal epithelium by electron microscopy (Dalton and Felix, 1954) and the rapidly succeeding papers showing a similar structure in kidney (Rhodin, 1954) and pancreas (Sjöstrand and Hanson, 1954) it has become routine to describe its structure in any electron microscopic study.

The classical silver and osmium impregnation methods have been applied to amoebae and other protozoa with conflicting results. Various structures have been regarded as the analogue of the Golgi material. Smyth (1944) and Pappas (1954) thought it to be the heavy spherical bodies; Brown (1930) apparently confused it with mitochondria (Andresen, 1956) or heavy spherical bodies (Mast and Doyle, 1935a). Mast and Doyle (1935a) found that if any Golgi material is present it is in the outer layer of heavy spherical bodies. Naessonov (1924) believed the contractile vacuole was homologous with Golgi material in several protozoa and Gatenby, Dalton and Felix (1955) found support for this in the EM.

Electron microscopy demonstrates a completely different structure with the features of metazoan Golgi apparatus (Cohen, 1957, Pappas, 1959) and it is now customary to regard this well defined organelle as the Golgi apparatus

(Schneider and Wohlfarth-Botterman, 1959). This study confirms previous findings and adds detail.

Observations (microgs. 36, 68, 43, 45)

Aggregations of membrane arranged in a highly characteristic manner are found throughout the cytoplasm of normal and experimental amoebae. Each mass is about 1 or 2 μ wide and consists of a stack of flattened slightly curved sacs or vesicles. The initial impression is of a stack of parallel membranes with adjacent membranes continuous at their periphery to form the walls of a closed sac.

The contents are indistinguishable from background cytoplasm. The periphery of each sac may be dilated to look like a pear-shaped vacuole in section. The sacs usually form a pyramid and those nearest the apex are the most dilated. Groups of small round vesicles occur near the apex.

After permanganate fixation, the individual membranes have a triple-layered structure with a total thickness of 90 Å similar to that of other cell membranes including the Golgi apparatus (Robertson, 1958, 1962) (microg. 43).

The small round vesicles found near the apex resemble the alpha particles. There is no direct evidence that they come from the Golgi membranes but in two examples taken after pinocytosis, discussed fully in section IV, there is

a more convincing relationship. The size of the vesicles in these cases (mean diameter $1,650 \text{ \AA} \pm \text{s.d. } 450 \text{ \AA}$) makes them indistinguishable on that basis from satellite vesicles of food vacuoles ($P = 0.75$) (section IV, appendix 4(i)).

Discussion

The Golgi apparatus now has an established structure associating three components (Pollister and Pollister, 1957, Dalton, 1961) large, empty vacuoles, parallel membranes and smaller vesicles. The structure described in amoebae bears a clear resemblance to the last two of these three. The structures believed in the past to be part of the Golgi apparatus in amoeba are, therefore, excluded. The small size and large number of the structures were probably factors in the failure to recognise them previously. No attempt has yet been made to demonstrate reduction of osmium or silver salts by this new organelle in amoebae.

The function of the Golgi apparatus is not clearly defined. Early light microscopic studies involving it in lipid transport and absorption (Krehl, 1890) are confirmed electron microscopically (Palay and Karlin, 1959, Dalton, 1961), and so is the part played in secretory activity (Bowen, 1929, Palay, 1958). Many authors have shown circumstantial electron microscopic evidence for the morphological part played in mucus secretion (Rhodin and Dalhamn, 1956, Palay, 1958).

Shearman and Muir, 1960, Bierring, 1962, Florey, 1962, Hayward and Johnston, 1962).

Chemically the Golgi region has been shown to contain acid phosphatase and phospholipids but no ribonucleic acid (Kuff and Dalton, 1960).

Only the possible association of small vesicles and Golgi apparatus in amoeba provides any clue to its function, but is by no means conclusive. The results obtained follow pinocytosis but cannot be unequivocally related to it. It must be borne in mind that Zelgel and Dalton (1962) believe that vesicles associated with the Golgi zone are fusing with it and not splitting away. The identity of the vesicles is doubtful. They are related in size to the satellites of food vacuoles and as a tentative hypothesis it can be suggested that in keeping with the role played in secretion in other cells, some of these vesicles pass from the Golgi zone to the food vacuoles. However, there is no supporting evidence, particularly of the source of enzyme protein.

FAT DROPLETS

Fat occurs as cytoplasmic droplets in amoebae (Mast and Doyle, 1935a, Andresen, 1956) which decrease in number during starvation (Mast and Doyle, 1935b, Cohen, 1957) but do not disappear completely after 3 weeks starvation (Holter and Zeuthen, 1948, unpublished personal observations).

The fat in the droplets originates in the food vacuoles. It is absorbed from digested food at a submicroscopic level (Mast and Doyle, 1935b). Amoebae can secrete lipases (Wilber, 1942).

In this material fat droplets are moderately osmiophilic spherical masses varying from 2.5 to 4 μ in diameter with a clear cut margin, sometimes marked with a slightly denser line (micrographs. 32, 39, 46). They lie free in the cytoplasm with no surrounding membrane. Small dense bodies usually found in association with membranes are sometimes seen attached to the outer surface of the fat droplets.

Except in perfectly sectioned material the fat droplets are liable to show "chatter", as light and dark bands at right angles to the direction of cutting, which results from cutting hard materials or using too obtuse a knife angle.

Fat droplets have not been found inside food vacuoles or other cytoplasmic fusion droplets in this material.

The small dense bodies found on the cytoplasmic surface of fat droplets are more usually associated with membranous structures and combined with the observations on centrifuged Pelomyxa they raise the possibility that fat droplets possess an outer membrane. Certain structures have been found (microgs.41,42) in Amoeba proteus which could be developing fat droplets within an outer membrane. However without the additional information provided by centrifugation these structures cannot definitely be distinguished from heavy spherical bodies. There is no other evidence of the origin of fat droplets. They have not been seen within food vacuoles or other structures. In one case (microg.50) a cytomembranous vesicle contains what appears to be a fat droplet but its exact nature is uncertain.

CRYSTALS

The cytoplasmic crystals of Amoeba proteus have been identified as triuret (carbonyldiurea) both by chemical and X-ray crystallographic methods (Griffin, 1960, Grunbaum, Møller and Thomas, 1959, Carlström and Møller, 1961). They probably form a partially stored, low molecular weight nitrogenous excretion product and constitute 40% of the nitrogen in a lipid-free amoebae (Grunbaum et al, 1959). The triuret is present as a stearic form of the stable triuret which can be formed in the laboratory but there are differences which cannot be explained on these grounds (Carlström and Møller, 1961) and attempts to duplicate the structure in vitro have failed.

Crystals occur as areas of complete translucency with an angular outline enclosed in complete outer membranes (microgs. 36, 40). The electron density is lower even than the embedding material alone and they must be holes in the section. The translucent area sometimes has a dark smudge at one edge (see heavy spherical bodies). The material between the outer membrane and the crystal negative is of the same density as the cytoplasm. The membrane has not been studied with higher resolution. Small dense bodies have not been

seen attached to the crystal membranes. From the study of centrifuged material in which vacuoles which gravitate to the crystal layer contain no crystals, it is deduced that "non-crystal bearing" crystal vacuoles exist. They presumably contain the mother liquor for crystal production. Some of the unidentified vacuoles of the cytoplasm may, therefore, be of this nature.

Discussion

The well documented chemical and physical analysis of amoeba crystals mentioned above leaves little to be gained from electron microscopy. The crystals survive fixation and embedding but disappear during screening, either in the microscope vacuum or because of the heating effect of the electron beam. The methods for examination of volatile substances (Bradley, 1961) could be applied only to mass preparations which are not available in this material.

The origin of the crystals is equally obscure from electron microscopy. The vacuole in which they occur has been said to be associated with the small dense granules usually found on plasma membrane (Greider, Kostir and Frajola 1958) but it is always quite free in this material. The high density of the crystals ($d = 1.74 \text{ gm/ml}$ (Grunbaum et al, 1959)) explains their centrifugal position in the Pelomyxa (section I). The ground substance of crystal

vacuoles is reported to be alkaline and to vary in consistency (Mast and Doyle,1935a). There is no evidence of crystal negatives in food vacuoles or associated with heavy spherical bodies to confirm the suggestions of their origin from food vacuoles or incorporation into heavy spherical bodies (Mast and Doyle,1935b).

SECTION III

THE FINE STRUCTURE OF BACTERIAL
COMPLEXES IN AMOEBA PROTEUS
(BRISTOL STRAIN)

THE FINE STRUCTURE OF BACTERIAL COMPLEXES
IN AMOEBA PROTEUS (BRISTOL STRAIN)

SECTION III

Intracellular bacteria are well known to occur in amoebae. Forms found in Pelomyxa pulustris have been studied bacteriologically (Keller, 1949, Leiner, Wohlfeil and Schmidt, 1951) and are undoubtedly symbionts concerned in the metabolism of the host. Zoochlorellae and fungi are also found in association with amoebae.

Amoeba proteus is not regularly associated with bacteria. However electron micrographs show structures similar to bacteria in some strains (Roth and Daniels, 1961). The Bristol strain, grown at the Carlsberg Laboratory since 1953 is heavily infected with bacteria of an unknown type (Chapman-Andresen, 1963). Other strains grown at the Laboratory under identical conditions also have a few bacteria but one (the "Adams" strain) has remained consistently free over a period of 4 years. The Bristol strain is very consistent in its response to experimental procedures and is therefore used at the Laboratory for studies on pinocytosis. It is a strong growing strain but shows no constant qualitative differences from the others.

Apart from the work of Roth and Daniels (1961) and mention of bacteria in food vacuoles of Pelomyxa (Brandt and Pappas, 1962) and Amoeba proteus (Mercer, 1959), there has been no electron microscopic study of intracellular

bacteria in amoebae.

The electron microscopic study of A. proteus Bristol strain shows a highly specific structure associated with apparently healthy bacteria which is quite distinct from the food vacuoles and may represent a reaction between amoeba and bacteria.

Physiological studies in support of these findings are in progress (Chapman-Andresen and Hayward, 1963). The bacteria always occur in circumscribed cytoplasmic masses which behave as individual cytoplasmic components.

Observations

The smallest type of structure containing bacteria is illustrated in micrograph 47. It consists of a single irregular membrane resembling the cytomembranes, completely enclosing material of the same electron density as the cytoplasm. One or more bacteria lie free in the vacuole with occasional small vesicular bodies between them. Such small vacuoles are quite rare.

Most of the bacteria occur inside membrane bound complexes, usually the largest single cytoplasmic components, up to 25 μ in diameter and unrelated topographically to any other structure (micrographs 46, 49, 50).

The general appearance of a large complex is seen in micrograph 49. The limiting membrane is wrinkled, irregular and of low electron density and resembles the cytomembranes more than the plasma membrane or food vacuole wall. There are no granules on its cytoplasmic surface. It is sometimes closely applied to the underlying bacteria.

Bacteria are distributed randomly throughout the complex. Their structure is described later.

The space between the bacteria contains a few small vesicles of about 1,000 Å diameter but is almost completely taken up by large membrane bound spaces of about 2 - 10 μ diameter which are now referred to as "inner" vacuoles. Their outlines sometimes show compression by

each other or by the bacteria. The inner vacuole walls are exactly similar to the limiting membrane of the whole complex but are always separate from it (microg.48).

After potassium permanganate fixation the triple layer or unit-membrane can be seen in the walls with a total thickness of 90 Å similar to that of the plasma membrane and Golgi membranes (microg.57). The qualitative difference between these membranes appears to be in density rather than thickness. The cytomembranes themselves have not been resolved into 3 layers. None of the membranes of the complexes stain with phosphotungstic acid solution. The contrast between limiting membrane and food vacuole wall is well shown in micrograph 46.

The unique features of the bacterial complexes lie in the contents of the inner vacuoles. The simplest contents consist of finely granular material of low or moderate electron density comparable with the background contents of the food vacuoles. Crenated fibrils sometimes run the whole width of the vacuole or lie in short bundles in the lumen (microg.49). When the fibrils are attached to the inner vacuole wall, they give it a ragged appearance. Organised structure is often superimposed on the granular ground substance.

Concentric parallel lamellae may lie inside the periphery or as a central core (microgs.51-53). Those at

the periphery take the form of curved, flattened sacs; with adjacent pairs of membranes continuous at their ends. The laminated central core often has 2 centres probably as a result of sectioning a folded or reniform body. The whole inner vacuole is often taken up by the lamellae round a central mass of soap-bubble-like material.

After potassium permanganate fixation, the central and peripheral parts of otherwise lamellated masses are arranged as numerous vermiform tubular structures (microgs. 55,56).

Individual lamellae are much denser after permanganate; each is 90 - 100 Å thick and can be resolved into three equal layers (microgs.58,59). The outer two layers are dense and the central core is more translucent. The space between adjacent lamellae i.e. that between electron dense outer layers, is very variable and may be up to 300 Å. The triple layered structure has also been detected, with the same overall thickness following osmium fixation.

Neighbouring lamellae of such a mass sometimes fuse to form a knot-like structure (microg.52) where 4 - 8 lamellae become confluent for a distance of about 200 Å.

Examination of a large number of bacterial complexes show a wide variety of lamellated bodies (microgs.60-63). The mass often has a shrunken appearance with an increased

electron density. There is a partial loss or collapse of lamellation and the irregular scalloped outline is separated by a translucent space from the limiting membrane. These shrunken masses have an added, granular component, scattered freely among the lamellae. The individual granules are dense, lacking regular structure and about 200 Å in diameter. They are never seen elsewhere in the bacterial complexes.

In several bacterial complexes one or more of the inner vacuoles is replaced by an irregular osmophilic mass enclosed by the typical inner vacuole membrane shown in micrographs 65 and 66. The substance of these masses is not homogeneous but has a finely reticulated background within which the appearance of a more gross ordered pattern is seen. Here and there are a few clumps of curved parallel lamellae similar to those more frequently seen in the inner vacuoles.

Structure of the bacteria

All the bacteria are similar morphologically and resemble those found in the food vacuoles. They do not resemble those seen in other Amoeba proteus by Roth and Daniels (1961).

They are circular in cross section (0.5 µ diameter) and reach a maximum length of about 3 - 5 µ. The outer cell wall is a thick, dense, single line usually smooth and about 200 Å thick and is constantly separated from the bacteri

cell contents by a slightly wider but indistinct translucent space. Sometimes the membrane is lifted further away from the surface and frequently many bacteria in one vacuole have pointed, blob-like processes projecting from the surface. There is no visible outer capsule.

Very little detail is visible in the bacterial cytoplasm. It is uniformly granular with a paler central nuclear zone. Many bacteria have a grape-like cluster of round, apparently empty vesicles and no visible nuclear zone.

PTA staining leads to a generally increased contrast but selective intensification of the outer wall and of the periphery of the translucent vesicles (microg.50). They stain intensely showing that they are not completely empty. No cytoplasmic membranes have been seen in the bacteria.

The morphology of the bacteria is relatively constant suggesting a single type or closely related forms. From the dimensions of the wall they are gram negative (Glauert,1962) and from their overall dimensions bacillar. The translucent droplets probably represent bacterial lipids (Wyss,Neumann and Socolofsky,1961) which tend to accumulate in resting cells exposed to an amenable environment.

Division has not been seen in the complexes though it has been identified in a food vacuole (microg.37).

Food vacuoles

Food vacuole structure is described in section II. Bacteria are commonly present and are indistinguishable from those of the complexes. Their presence in Pelomyxa carolinensis has been noted, (Brandt and Pappas, 1962), but it is not possible to confirm that they are a criterion for the identification of food vacuoles or defaecation droplets. None of the food vacuoles of centrifuged Pelomyxa carolinensis contained bacteria and many of those of Amoeba proteus were also free. Prescott (1959) has shown that nucleic acid precursors are incorporated into food vacuoles probably by living micro-organisms.

Discussion

1. Origin of the bacteria.

The amoeba is normally fed on Tetrahymena sp. which are raised axenically, i.e. in a defined medium with no other organisms, but not under sterile conditions. Bacteria may occur as chance contaminants which Tetrahymena, as a bacteria feeder, would ingest.

Amoebae themselves do not normally live on bacteria but will ingest them (Kudo, 1946). Ciliates containing apparently healthy bacteria can be seen in electron micrographs of amoebae (Horysko and Roslansky, 1959). Roth and Daniels (1961) believe bacteria to be ingested during spontaneous pinocytosis.

The presence of bacteria in food vacuoles suggests they enter by one of these mechanisms and since they look healthy, are not degenerate and even divide, they must survive the enzymic environment of the food vacuole. Some "food vacuoles" in fact contain nothing but bacteria.

2. Type of bacteria

Apart from suggesting they are gram negative bacilli which contain lipid nothing can be said about their classification.

3. Nature of bacterial complexes

The complexes are quite distinct from food vacuoles and no possible intermediary has been found. The walls of the complexes are similar in structure to the cytomembranes.

It is well established in electron microscopy that phospholipids and lipoproteins exist as lamellated bodies (Geren and Schmitt, 1953, Stoekenius, 1959, Robertson, 1960, Stoekenius, Schulman and Prince, 1960, Finean, 1961) similar to those found in this study:-

- a. in the inner vacuoles of bacterial complexes
- b. in the food vacuoles
- c. in pinocytosis complexes (section IV)
- d. free in the cytoplasm of experimental amoebae (section IV).

X-ray diffraction (Bear, Palmer and Schmitt, 1941, Finean, 1961) combined with knowledge of their general structure shows

that phospholipids exist as bimolecular leaflets with the hydrophilic polar ends of the molecules pointing in one direction and the hydrophobic non-polar fatty acid chains in the other.

Electron microscopy shows that osmium tetroxide and potassium permanganate fixation preserve the bimolecular layers which appear as alternating light and dark bands. Pure phospholipid gives a repeating unit of about 40 Å by X-ray diffraction (Bear, Palmer and Schmitt, 1941) and hydrated phospholipid gives a period of 40 - 50 Å in the electron microscope (Stoekenius, 1959, Robertson, 1960).

Whether the osmiophilic dark band represents the reaction of the fixative with fatty acid chains or with the polar part of the molecule was initially doubtful. Stoekenius (1959) was able to argue that each osmiophilic layer represented the projecting unsaturated fatty acid residues. However, further detailed work cast doubt on this finding. Stoekenius, Schulman and Prince (1960) in a revised argument showed that synthetic phospholipids with fully saturated fatty acid chains which should not react with OsO_4 give the same pattern, a fact already demonstrated by Finean (1959). Furthermore, water penetrates the complex at the osmiophilic band. Protein added to a phospholipid is adsorbed on the bimolecular

leaflet at the osmiophilic layer which increases in thickness (Revel, Ito and Fawcett, 1958), further suggesting that it represents the polarised end of the lipid molecules. Protein will not penetrate beyond the most superficial layer of phospholipid, and proteolipid masses have the same spacing characteristics as pure phospholipids (Finean, 1961).

There is now little doubt that osmiophilia is the feature of the polarised part of the molecule but it remains possible that it results from migration of an ionised reaction product between osmic acid and non-saturated fatty acid chains (Finean, 1961).

Naturally occurring lamellar systems have been described (Bradbury and Meek, 1958, Chou and Meek, 1959, Walker, 1960, Miller, 1960, Mercer, 1961, Carr and Carr, 1962) and a number of these have been correlated histochemically with sites of phospholipid deposition (Chou and Meek, 1958, Carr and Carr, 1962). The best known example of a lamellar system, the myelin of central and peripheral nerve fibres, is a complex mixture of lipids, including phospholipids, as well as cholesterol and cerebrosides, protein and carbohydrates. It has received detailed analysis (Finean, 1961) but its structure is still uncertain. The repeating unit of naturally occurring lamellae is not always recorded but the width of the lamellae has been used in an attempt

to distinguish between phospholipids and lipoproteins in the protein absorption droplets of kidney tubules (Thoenes,1962). As mentioned above such a distinction is not fully justified by the study of prepared systems (Finean,1961).

Plasma membrane is a complex of protein and phospholipid (Engström and Finean,1958) and in confirmation of a model system (Davson and Danielli,1952) the unit membrane structure of many cell membranes (Robertson,1958, 1960) can be considered a special case of bimolecular layering with protein adsorbed on the outer hydrophilic ends of phospholipid molecules. Lamellated bodies are particularly common in granules resulting from phagocytosis or pinocytosis (Karrer,1960c; Miller,1960, Walker,1960, Hayward,1961) and can be attributed to the amalgamation of lipoproteins from ingestion of substantial amounts of plasma membrane. Similar masses occur in amoebae (section IV

The lamellae of food vacuoles show close-packing and a repeating unit of approximately 90 Å between the centres of 40 Å wide osmiophilic bands. The picture obtained is at the limit of resolution of this study and could be interpreted in two ways. The single osmiophilic band might represent the whole bimolecular leaflet and be potentially resolved into unit dimensions. Its total width of 40 Å is compatible with such a structure. The translucent layer then represents water.

Alternatively the translucent layer may be regarded as the fatty acid fraction of a pure phospholipid and the osmophilic bands as opposed polarised fraction.

The figures given by Stoekenius, Schulman and Prince (1960) give a smaller width for the hydrophobic layer which would in this case be 50 Å. Furthermore this layer is not quite constant in width as it should be if it represented part of a molecule.

The picture is, therefore, interpreted as a hydrated phospholipid in which unit dimension of pure phospholipid leaflets have not been resolved.

In the case of bacterial complexes the interpretation is simpler. Micrograph 59 clearly represents bimolecular leaflets with a total width of approximately 90 - 100 Å separated by much wider spaces presumably occupied largely by water. The light central band is about 30 Å wide but its exact dimensions are impossible to determine. The dimensions of each leaflet are therefore incompatible with a pure phospholipid but on the findings of Revel, Ito and Fawcett (1958) may be regarded as owing their extra width to a layer of adsorbed protein. Only Finean's observation (1961) that proteolipids in vitro have the dimensions of phospholipids, casts doubt on the deduction.

In view of the relationship with bacteria it is of

considerable interest to note that the only observations on extracted lipopolysaccharides of bacterial origin show a lamellar spacing of 85 Å which is not too far removed from this one (Finean, 1961). Lipopolysaccharides have never been encountered in electron micrographs in natural conditions.

It is tentatively believed, therefore, that food vacuoles contain pure phospholipids occurring as moderately hydrated close-packed masses whereas bacterial complexes contain highly hydrated lipoproteins.

The modifications of the lamellated bodies may be the results of varying degrees of hydration, possibly a result of local variations in tonicity in vivo. The fact that they are not seen after permanganate fixation is in agreement with Finean's finding (1961) that that fixative may result in some rehydration. There is a striking similarity between micrograph 61 and figure 10 of Finean showing unfixed myelin after dehydration in alcohol-ether.

The dense granulation is unidentified although Revel, Ito and Fawcett (1958) found similar granules in artificial phospholipid preparations.

The source of the protein-phospholipid complex is not known. Lamellated bodies occur in cases of cell degeneration including tubercle infection (Cedergren, 1957).

It seems likely that bacteria were initially

contained within plasma membrane bound vacuoles. For unknown reasons a new membrane possibly of cytomembrane origin is deposited around such a vacuole whose wall deteriorates into the stable myelin form of lipoprotein. Membranes of cytomembrane origin are deposited around massive pinocytosis channels (section IV) and lamellated bodies occur in the same specimens.

The evolution of a lamellated body from an empty inner vacuole, with progressive laying down of lamellae to a highly hydrated complex mass with subsequent dehydration and collapse of lamella, can only be hypothetical.

The part played by the bacteria in the metabolism of the amoeba is obscure. Those of Pelomyxa palustris (Leiner et al, 1951) lie free in the cytoplasm (by light microscopy) and follow a complex sequence of movements which may be associated with polysaccharide metabolism.

The bacteria of A. proteus are always isolated from the cytoplasm. Roth and Daniels (1961) point out the possible confusion which may arise in studies of nucleic acid synthesis in amoeba infected with bacteria. Plaut (1960) showed DNA synthesis in the cytoplasm claiming micro-organisms to be absent and Rabinowitz and Plaut (1962) associated the DNA with a small granular component of cytoplasm with definite centrifugation characteristics but they reserve the possibility of an infective organism.

Electron microscopy of the specimens used is essential to exclude bacteria, yeasts or viruses.

Cytoplasmic incorporation of ribonucleic acid precursors in the absence of the nucleus has been reported (Plaut and Rustad, 1956, 1957) but also denied (Prescott, 1959). It does occur in the presence of the nucleus (Prescott, 1957). Mast and Doyle (1935a) suspect the presence of infective organisms in Amoeba proteus, Mercer (1959) raised the same possibility. Daniels and Roth (1961) suggest there may be some in P. illinoiensis and Prescott (1959) and Brachet (1960) mention the possibility following biochemical studies in Amoeba proteus.

This material further suggests that in the event of physiological studies on lipid or phosphate metabolism the presence of bacteria must be taken into account. As Roth and Daniels (1961) point out nucleic acid studies should be carried out on uninfected stock carefully monitored by electron microscopy.

SECTION IV

FINE STRUCTURAL CHANGES FOLLOWING
THE INDUCTION OF PINOCYTOSIS IN
AMOEBIA PROTEUS WITH ALCIAN BLUE
SODIUM CHLORIDE AND ALBUMIN.

FINE STRUCTURAL CHANGES FOLLOWING THE INDUCTION OF
PINOCYTOSIS IN AMOEBA PROTEUS WITH ALCIAN BLUE
SODIUM CHLORIDE AND ALBUMIN SECTION IV

1. Pinocytosis induced by alcian blue.

As soon as the amoebae are immersed in alcian blue solution, the outer membrane is stained blue and the staining shows up clearly when the organisms are still immersed, suggesting that, as with other inducers (Schumaker, 1958), the dye is concentrated on the surface.

The staining occurs equally well if the amoebae and the solution are at 4°C before immersion.

After osmium fixation in the electron microscope, the alcian blue treated plasmalemma has the appearance shown in micrograph 67. Instead of the outer filamentous layer there is a series of rounded moderately dense masses with no visible connection with the membrane or each other. A section tangential to the surface shows that the whole membrane is covered by such masses (microg. 70). The change is quite constant and represents the irreversible reaction between alcian blue and filamentous mucopolysaccharide. Each globule represents an amalgamated group of filaments.

The space between globules and membrane probably indicates the presence of an invisible cement substance since the globules show no tendency to separate from the membrane before ingestion.

Simultaneously with the change in the filaments the cytoplasmic ground substance underlying the plasmalemma shows a patchy coarse granulation for a depth of 1-1.5 μ .

The change occurs only in localised areas and is sometimes accompanied by pleating of the adjacent plasmalemma (microg.68). The membrane forms shallow ripples dipping down into the cytoplasm. There is no visible change in the plasmalemma at these points and normal cytoplasmic organelles are found close by. Such folds are interpreted as incipient channels.

After five minutes immersion in alcian blue solution, many channels are seen by light microscopy and are easily found in the electron micrographs.

Each channel can be divided into three parts. The opening is funnel-shaped and is often surrounded by granular cytoplasm (microg.69). It leads to an elongated neck which may be up to thirty microns in length (in section) and terminates in a bulb-like expansion (microg.71). The walls of the channel are identical with plasmalemma and are often as little as 1,000 Å apart at the neck. The channel contains the globules of alcian blue mucoid complex (ABM) either still attached to the walls or lying free in the lumen. Small dense bodies (section II) are often seen on the cytoplasmic surface of the plasma membrane.

There are few, if any, cytoplasmic organelles in the vicinity of the necks of these channels. Occasional mitochondria are seen near the walls, but they are too

infrequent to conclude a specific relationship. Granular cytoplasm is found in restricted collar-like regions along the walls of the neck (microg.71). It is finer and more even and compact than that already found nearer the surface. The amoebae are rather fragile during pinocytosis (Chapman-Andresen and Dick, 1961a) and slight damage sometimes occurs particularly near channels. On these occasions the cytoplasm splits away from the plasma membrane but the granular material stays firmly attached to the membrane or the channel wall.

Dense debris is sometimes present in the cytoplasm and accumulates along the edge of the granular zone as if it had been swept in front of the developing granular material. The impression given by the micrographs is that the granular cytoplasm represents a change in physical properties of the matrix and that it is more firmly adherent to the plasma membrane. There is no evidence about its chemical nature.

The distal end of a channel is shown in micrographs 72 and 76a. It is roughly pear-shaped and has terminal finger-like protrusions extending into the cytoplasm. The lumen contains globules of ABM completely separated from the plasma membrane as well as other unidentified debris. All the bulbous ends of channels examined have a granular cap of cytoplasm through which

the extensions of the lumen project. Normal cytoplasmic constituents are found beyond the granular material but not closely related to the channel.

The granular cytoplasm does not always cover the protrusions of the lumen giving the impression that the channel might extend through the granular cap so that as the channel lengthens it would acquire a circumscribed granular collar like that described above.

The channels usually appear to be cut longitudinally but occasional transverse sections show them to be flattened in one dimension so that the longitudinal sections may be comparatively oblique (microg. 73, 74). With this reservation in mind, the dimensions of a mature channel are approximately 30 μ long, and 0.2 - 2 μ by 4 - 10 μ in section.

The subsequent development in the light microscope is marked by disappearance of channels connected with the plasma membrane and the formation of a complex central blue mass (Chapman-Andresen, 1963). It proves to be a difficult stage to interpret from electron micrographs.

At first the channels develop extensive intercommunicating processes into the surrounding cytoplasm (microg. 75). The main channels divide, dilate and finally break up into short closed lengths. It is impossible to show a representative picture of the peak of development. Most of the cytoplasm involved consists of attenuated

processes, separated by dilated channels, some of which contain clumps of shed ABM. There is obviously a great deal of cytoplasmic movement about which static micrographs give little information.

After being left for an hour in Pringsheim's solution to recover, the amoebae show a more definitive pattern in which three structures remain as a result of fragmentation and fusion of channels and droplets.

It should perhaps be noted that the sequence observed by Mast and Doyle (1934) of small droplets breaking away from the end of developing channels has not been seen. The thinness of the sections combined with the absence of movement in the micrographs might make it difficult to detect such a process.

In most of the cytoplasm, there are short closed channel lengths forming irregular elongated vesicles which often branch and interconnect (microg.76). The walls of the vesicles retain the characteristics of the plasma membrane and the channel walls, and are more dense and regular than the cytoplasmic membranes. A triple membrane structure has not, unfortunately, been demonstrated in this material but the vesicle walls are intensified by PTA. An average vesicle of this type is 0.5 - 2 microns in diameter and about 12 microns long. The vesicle contents, like those of the channel necks, consist of ABM either

attached to the inner surface of the membrane or lying free. Granular cytoplasm is not seen in the vicinity and small droplets or mitochondria are not found near the vesicles in significant numbers.

In addition to the channel lengths there are two types of large round droplets. The first is spherical, approximately 6 microns in diameter with slightly irregular walls freely studded with small dense bodies on the outer (i.e. cytoplasmic) surface (microg.78). The walls stain selectively with PTA and the process removes the small dense bodies leaving translucent spaces (microg.79). From their general appearance and staining reaction the walls of the vacuole are probably derived from the channel walls and hence from the plasmalemma. The contents consist almost entirely of ABM with no interspersed membranes. Many of the globules of ABM retain a linear arrangement as if still attached to membrane with nothing visibly connecting them. The impression is again that a cement substance of low osmiophilia and electron density is present. The contents of the droplet are separated from the wall by an empty space.

The second type of droplet remaining after pinocytosis is much larger, up to 15 μ in diameter and characteristically contains both membranes and ABM (microg.80)

The walls stain with PTA and are freely studded with small dense bodies. In both types of droplet, the walls exhibit more small dense bodies than an equivalent length of normal plasma membrane. The small dense bodies preferentially accumulate on the surface of fusion droplets.

The central mass of the large fusion droplets is roughly spherical with broad irregular space separating it from the outer membrane. It consists of fragmented membranes lying in bundles between loose clumps of ABM often arranged as if still attached to the channel walls.

Apart from the large fusion droplets, ingested plasma membrane in the form of intact channels containing ABM is found in other bodies. Micrograph 77 shows a body consisting of a continuous outer membrane enclosing an intact channel lying, free of cytoplasm, in the lumen. Both outer membrane and channel wall stain with PTA. Such a droplet may well be an early stage of development of a large fusion droplet formed by progressive addition of further channels and fragmentation of their walls.

The peripheral space of large fusion droplets contains faintly granular material and a few well formed lamellated bodies which are moderately dense and loosely arranged in an annulate form. Although they have not been studied at a higher resolution they are similar to

the lamellated masses of food vacuoles and are taken to indicate the presence of colloidal phospholipids or lipoproteins. Lamellated bodies were found in pinocytotic vesicles by Nachmias and Marshall (1961) but no interpretation was put forward. There was no membrane in the central fusion masses in such cases.

Two or more complex droplets sometimes fuse so that extra large complexes form, in which the contents remain as separate spheres without being separately membrane bound. The large complexes correspond to the "mulberry-shaped" vacuoles of Chapman-Andresen (1963).

The subsequent fate of the large droplet is not known. In view of the decrease in alcian blue vacuoles with time (Chapman-Andresen, 1963) it is assumed that they form defaecation droplets and are progressively eliminated.

None of the structures resulting from alcian blue induction are found to be related to small cytoplasmic vesicles in the way shown by Chapman-Andresen and Nilsson (1960), Nachmias and Marshall (1961) and Brandt and Pappas (1962). There is no evidence of micropinocytosis.

Toxic manifestations. In those amoebae subjected to alcian blue at pH 4.8 for three minutes, extremely vigorous pinocytosis is induced. At least 25% of a sample put aside for observati

died within five days but the remainder seemed to be healthy.

Following the initial immersion there was a prolonged period of inertia with a distended paralysed contractile vacuole. The amoebae remained smoothly spherical and streaming was beginning at the end of the experiment i.e. 6 hours after immersion.

The early changes in fine structure were similar to those described for amoebae immersed for two minutes at pH 5.8. Later stages showed significant differences probably as a result of ingestion of large quantities of plasma membrane.

After three hours, a large number of closed channel lengths, similar to those described above are grouped together and partially or completely surrounded by a separate membrane (microgs.82,83). The new membrane, which is distinctly different in texture to the channel walls and more closely resembles the cytomembranes is usually double and separated from the channels by a narrow band of cytoplasm. Where the channels are not completely invested the two layers of the new membrane are continuous at the free end, so as to form a flat, elongated sac. Occasionally the complete membrane is only of single thickness.

The origin of this membrane is not known but it

might be formed by progressive alignment of flattened cytomembranous sacs round the periphery of the mass and fusion to form a continuous double membrane. Alternatively the membrane might condense directly from the cytoplasmic ground substance. It provides a striking example of separation of ingested material from the rest of the cytoplasm. There is reason to believe that the cytoplasm contains an excessive amount of phospholipid in these specimens.

The new membrane may of course represent a terminal phenomenon and it has not been possible to follow the subsequent fate of material isolated in this way.

The amoebae show other signs of structural damage in heavily induced specimens. The mitochondria are contracted with an irregular outline and close-set tubules showing a loss of intertubular matrix (microg.93,94). They are similar in structure to those found in cytoplasmic sequestration (below) and presumably result from toxic damage or the failure of normal streaming to bring them into contact with substrate material.

An additional feature, never seen in normal amoebae is the presence of large masses of osmiophilic lamellated material lying free in the cytoplasm with no limiting membrane (microgs.93,94). From the remarks made elsewhere it can be seen that here again is a build up of cytoplasmic

phospholipid or lipoprotein in excess of normal, leading to the formation of visible aggregations.

The presence of lamellae in pinocytosis droplets as in food vacuoles means the presence of phospholipids. The only structure present in the droplets which contains phospholipid is the ingested plasma membrane and the finding implies that this membrane is broken down presumably by proteolytic enzymes. The free lamellae of the cytoplasm also suggest an excessive cytoplasmic build up of phospholipid beyond the limited capacity of the amoeba metabolism.

No changes are found in any other cytoplasmic components as a result of pinocytosis. The ingested material is limited to fusion droplets and channels and not found in small cytoplasmic vesicles or contractile vacuoles as in the experiments of Brandt and Pappas (1962).

2. Pinocytosis induced by sodium chloride and albumin

A small amount of material from these experiments is included to compare the results with those from alcian blue. The specific changes found in those experiments, especially the granular change in the cytoplasm might be attributed to the unphysiological nature of the inducer. Although sodium chloride and albumin do not occur in the normal amoeba habitat in the concentrations used as inducers they resemble the normal cytoplasmic constituents more than

does alcian blue.

Satisfactory micrographs of early channels were not obtained using sodium chloride or albumin and the remaining findings are conveniently considered as follows:-
The outer layer of the plasma membrane. The filamentous layer is not always visible with the methods employed in normal amoebae (section II). With a few exceptions, the plasmalemma after sodium chloride induction is also devoid of filaments with neither membrane nor channels showing any evidence of the outer mucoid material.

After albumin the filaments are usually clearly visible as thin parallel lines at right angles to the membrane (microg.87). Channels too are lined by a filamentous layer. It would be expected from other experiments (Chapman-Andresen and Holtzer, 1960) that the Pringsheim's solution used for washing (pH 6.8) would have eluted the adsorbed albumin but the time for washing may have been too short. Certainly the filaments are either made more electron dense or stabilised against preparatory treatments by immersion in albumin, either because the protein is still present or because it produces a physical change in the filament structure. There is no suggestion of the globules found after alcian blue.

Granular cytoplasm. At the stage of maximum pinocytosis, a complicated mass of channels is present and much of the intervening cytoplasm shows the dense granular change already described (microgs. 97-102). The relationship of such modified cytoplasm to the channels is much less clear cut than in the simple early channels of alcian blue but as in the later stages of induction with the dye there is a great deal of movement in the region of channel development, which probably disturbs the more simple arrangement. Granular cytoplasm is, therefore, a clear feature of pinocytosis with all three inducers. It is occasionally seen in later stages either round fragmented channels or fusion droplets notably after albumin (microg. 91). The channels in sodium chloride induced specimens are freely associated with mitochondria of normal structure but the association is not noticeable after albumin.

Structure of fusion droplets. Large fusion droplets occur following sodium chloride induction but contain only membrane fragments presumably because the filamentous material, ill-defined on the membranes and usually absent from the channels has completely disappeared (microg.90). There are a few lamellated bodies in the peripheral space of fusion droplets similar to those already described.

Some droplets are closely associated with small round cytoplasmic vesicles in the same way that normal food vacuoles have satellite vesicles (section II). Nachmias and Marshall (1961) have shown micropinocytosis vesicles after ferritin induction. There is again no evidence of small vesicles near channels like those shown by Chapman-Andresen and Nilsson (1960).

Channels induced by albumin have visible filamentous contents and fusion droplets have a central amorphous, moderately electron dense mass presumably derived from the mucoid layer and adsorbed protein (microgs. 88, 89). The filamentous nature is lost on fusion. Occasional droplets have a central mass of fragmented membranes.

3. Cytoplasmic sequestration

At some stage after all the pinocytosis inducers used, structures like that in micrograph 95 can be found.

A fragment of cytoplasm, usually including a mitochondrion is enclosed and separated or "sequestered" from the rest of the organism by two concentric membranes.

The limiting membranes are seldom parallel and the inner usually has marked folds or invaginations. The space between the two is empty.

The contents may include other organelles; that in micrograph 96 incorporates a fat droplet and Golgi

membranes. The trapped mitochondrion may be in various stages of deterioration. It usually has a contracted outline and close packed tubules with loss of intertubular matrix. Some of the mitochondria show blebbing of the outer limiting membrane. The degree of damage is not related to the time elapsing since induction. There may be severe damage after ten minutes but some examples are still intact after two hours.

Serial sections were obtained from one amoeba ten minutes after immersion in sodium chloride solution at the height of pinocytotic activity (microgs.97-102). A mitochondrion with definite signs of damage can be seen surrounded by two concentric, closely applied membranes. Sections from further along the series indicate that the space between the two membranes is probably continuous with a pinocytotic channel (in which, contrary to the general appearance, a filamentous layer is visible). It appears, therefore, that the membranes of a sequestration are derived from the walls of a channel. During the intense churning activity in such an area it is quite conceivable that a tongue of cytoplasm, in this case containing a mitochondrion is pushed or drawn into a channel so that opposing walls are pushed close together. Subsequent breakdown of the channel leads to isolation of an island of cytoplasm by two continuous membranes. The proposed

sequence of events is shown diagrammatically in diagram 6. The structural damage to the mitochondrion must be due to its isolation and deprivation of essential substrates or oxygen in the cytoplasm. It resembles the damage found after excessive pinocytosis and similarly may reflect the effective absence of streaming. The frequency with which mitochondria are involved appears to provide support for the relationship of mitochondria with pinocytosis channels at the stage of maximum development.

It is tempting to relate sequestration with the fusion of mitochondria and pinocytosis droplets reported by Gey et al (1955) but this material suggests invagination into channels rather than fusion with droplets. Complexes containing mitochondria are found in the epithelium of new born mammalian small intestine (Moe and Behnke, 1962) the cells of which display vigorous pinocytosis (Clark, 1959).

The term sequestration has been independently applied to isolation of mitochondria in pancreatic acinar cells after poisoning with an amino-acid analogue (Hruban, Swift and Wissler, 1962) and "sequestered" material in pulmonary adenoma cells was believed to be trapped in tubes of endoplasmic reticulum (Svoboda, 1962).

4 Golgi apparatus

During and after pinocytosis amoebae contain normal Golgi apparatus, described in section II. In two examples,

shortly after pinocytosis induction, there is a more definite relationship between membranes and small vesicles. In micrograph 103 there is an example with the usual pyramid of curved flattened sacs. Near the apex the sacs show localised dilatations which become more marked as the apex is approached until they are almost indistinguishable from vesicles lying free near the apex. A second example shows a larger number of small vesicles than is usually seen and they have a mean diameter of $1,650 \text{ \AA} \pm \text{s.d. } 450 \text{ \AA}$ (appendix 4) and on the basis of size alone are indistinguishable from the food vacuole satellites ($P = 0.75$).

The close association of vesicles with the Golgi apparatus suggests that they are derived from dilatations of the membranous sacs. The acid test of identical unit membrane structure of the walls has not been achieved, probably because the section thickness (approximately $1,000 \text{ \AA}$) is comparable with the diameter of the vesicles.

The mechanism of progressive dilatation of the Golgi membranes has been repeatedly postulated for the storage of mucus (references see section II) in mammalian goblet cells. The present observations merely raise the possibility that some of the small vesicles of the cytoplasm arise in the Golgi apparatus. Zeigel and Dalton (1962) believe that small vesicles in protein secreting cells are being incorporated into the Golgi membranes.

So far the association of vesicles and Golgi membranes is found after pinocytosis but the results are so few that they could represent a rare normal occurrence and not a response to pinocytosis. If vesicles were produced intermittently with long periods of quiescence, there are so many Golgi zones in each amoeba that an active one need be found only by chance.

5 Discussion

Discussion of Pinocytosis in Amoeba proteus: The outer or filamentous layer of plasmalemma of amoebae is present in micrographs in this and other work. It contains acid mucopolysaccharides and Marshall, Schumaker and Brandt (1959) suggest the surface may present a series of negatively charged groups to the outer medium. The charge potentials on amoeba plasma membrane have been measured (Bingley and Thompson, 1962, Bingley, Bell and Jeon, 1962) and are negative in normal culture medium but decrease to almost zero in certain salt solutions. It is this outer layer which is most closely related to the freshly introduced inducer.

The initial event in induction of pinocytosis is believed to be the adsorption of the inducer on to the cell membrane complex (Brandt, 1958, Nachmias and Marshall, 1961, Brandt and Pappas, 1961, 1962). The systematic study of inducers (Chapman-Andresen, 1963) shows that only

charged molecules are effective. The pH of protein solutions upon which the ionisation of the molecule depends is critical for their action.

Chapman-Andresen (1963) attempts to divide inducers into three groups on the basis of the reversibility of their adsorption to the outer membrane. In the first group, ionised salts, the presence of the inducer on the membrane has not been demonstrated and the inducing action ceases immediately the solution is removed (Chapman-Andresen and Prescott, 1956). The second group consists of proteins and amino-acids, their presence on the membrane can be demonstrated by fluorescent labelling (Brandt, 1958, Schumaker, 1958) or electron microscopy (Nachmias and Marshall, 1961) and they remain adsorbed when the solution is removed providing the pH does not change (Chapman-Andresen and Holtzer, 1960). Some of the basic dyes belong to this group. Their initial activity depends on the ionisation of the molecule, that is on the pH of solution and persists so long as the pH does not change and reduce the ionisation. Alcian blue constitutes a third group in which the plasma membrane is strongly and irreversibly stained.

The difference between the three groups and Chapman-Andresen's hypothesis of the mechanisms are partially substantiated by this study. She believes that

salts dissolve the filamentous layer, that proteins produce a reversible change in its structure like that which occurs in mucus and that alcian blue forms a stable compound. Dissolution or absence of the filamentous layer after sodium chloride treatment cannot be justifiably deduced as the layer is poorly preserved by routine methods. Albumin treated specimens however, always show a prominent filamentous layer even though bovine plasma albumin should be eluted from the surface by Pringsheim's solution at pH 6.8 (Chapman-Andresen and Holtzer, 1960). The improved preservation may, therefore, be as much due to a change in structure of the mucoid as to residual protein. The complex globules with alcian blue are treated by the amoeba as an insoluble, indigestible mass which is segregated into defaecation droplets. Alcian blue reacts with acidic groups by a stable salt linkage (Pearse, 1960) so that the general pattern of negatively charged groups on the plasmalemma is verified but the absence of a published formula for alcian blue makes further analysis impossible.

Latta (1962) has suggested that "staining" with phosphotungstic acid (PTA) is specific for positively charged groups. The work on pinocytosis does not confirm their presence on the filaments, but there is no evidence that inducers are adsorbed on the plasmalemma proper which

shows strong PTA staining. The routine resolution of this study is insufficient to confirm the differential thickening of the outermost layer of the unit membrane structure shown by Latta in kidney. The staining of the two elements of the whole plasma membrane may differ in mechanism.

The general study of polysaccharide layers on cell walls leads to the conclusion that they may be responsible in part at least for the overall negative charge (Eyler et al, 1962, Dorfman, 1963). The negative charge may often be due to terminal carboxyl groups of sialic acid in the polysaccharide molecule (Dorfman, 1963) and be associated with the precipitation of acid mucopolysaccharides which occurs with metallic cations and cationic dyes (Szirmai, 1963) as well as alcian blue and positively charged protein molecules. The salt complex with the cations of the first group of inducers may well be water soluble in contrast to that formed with heavy metal fixatives.

The initial adsorption, which is dependant on factors influencing the physical state of the inducer (i.e. pH, concentration) does not depend on the metabolism of the amoeba and occurs at low temperatures (Schumaker, 1958, Nachmias and Marshall, 1961) and in the presence of metabolic inhibitors. The second stage of channel initiation is

undoubtedly dependant on metabolic factors. It does not occur until the temperature is raised to normal levels or in the presence of metabolic inhibitors. Sbarra and Karnovsky(1959) have shown a strong correlation between oxidative phosphorylation and phagocytosis in leucocytes.

Brandt (1958) suggests that the plasmalemma becomes firmly attached to underlying gelated cytoplasm. A combination of cytoplasmic flow past the point of attachment and gel retraction produces a channel with its tip at the point of attachment. Chapman-Andresen (1963) draws attention to the fact that active pinocytosis can be induced in anucleate amoebae (Chapman-Andresen and Prescott,1956) in which pseudopod formation and cytoplasmic flow do not occur.

The local granular change in the cytoplasm round alcian blue channels is significant and has not previously been described. Routine examination of normal amoebae has not demonstrated evidence of sol to gel transformation. Granulation has been reported in cysts of some protozoa (Wohlfarth-Botterman and Schneider,1958, Schuster,1962) and has been attributed to gelation and dehydration. Granulation occurs in amoeba cytoplasm after decompression from high pressures (Landau and Thibodeau,1962) but because of different fixation (potassium permanganate) and

embedding (methacrylate) direct comparison with the micrographs is difficult. It is known that gel formation requires energy and that gel is potentially contractile (Landau, 1959). Mast and Doyle (1934) thought pinocytosis was a response to osmotic dehydration but Chapman-Andresen and Dick (1961a) were unable to find a constant volume decrease in Pelomyxa during pinocytosis even though the overall volumes in inducing solutions were less than in non-inducers with the same osmotic pressure. Contractile vacuole activity is inhibited by pinocytosis (Kitching, 1956a, Chapman-Andresen, 1963) but the mechanism is unknown.

The granular change of pinocytosis has been noted round certain channels of unknown origin in the centripetal zone of centrifuged Pelomyxa carolinensis. The origin of such channels is discussed in section I. The association with granular cytoplasm suggests they probably arise as a result of pinocytosis or a related phenomenon.

The patchy coarse granulation immediately under the surface plasmalemma contrasts with the firm finer material round the channel necks. Whatever its nature it is clearly accompanied by a firm anchoring of cytoplasm to plasmalemma. The fragility of pinocytosing amoebae (Chapman-Andresen and Dick, 1961a) results in cytoplasmic

splitting during preparation and this occurs between normal and granular cytoplasm leaving the granular area attached to the membrane (microg.71). The wrinkling of the plasmalemma suggests local contraction of underlying cytoplasm. Two of the elements in Brandt's hypothesis (1958) are therefore confirmed and further retraction combined with cytoplasmic flow past the site of attachment would lead to channel formation. There is no evidence of the mechanism linking adsorption with this cytoplasmic change.

After ingestion, the filamentous layer and its adsorbed alcian blue referred to for convenience as ABM (alcian blue-mucoid) separates from the plasmalemma proper. The separation is comparable to that of adsorbed "Thorotrast" described by Brandt and Pappas (1961) before ingestion which was thought to be purely mechanical. It is quite distinct from the shedding of ferritin from the filaments noted by Nachmias and Marshall (1961) after ingestion which represents reversal of protein adsorption by pH change. Alcian blue is not separated from the filaments under any circumstances.

Examination of fusion droplets from albumin ingestion suggests that some protein persists. The central mass is fairly osmophilic and loses its filamentous nature. In this case separation of bovine plasma albumin from the

filaments does not occur after ingestion. Since the reversal of ferritin adsorption occurs at pH 5 and above (Nachmias and Marshall, 1961) and bovine plasma albumin does not evoke a pinocytotic effect above pH 5.5 and its adsorption is reversed at pH 6.5 or less (Chapman-Andresen, 1963, Chapman-Andresen and Holtzer, 1960) it can be suggested that the pH of the vacuoles rises to between 5 and 6. This method of measuring pH change, while not necessarily accurately applied here since eluted albumin might be rapidly absorbed or dissolved during preparation, is potentially a useful tool.

The change in pH is reminiscent of that which occurs in food vacuoles (Mast, 1942). Food vacuoles initially shrink and since in other protozoa the shrinkage is prevented by a hypertonic medium (Kitching, 1956b) it is probably the result of osmotic dehydration. At the same time the pH falls, either as a result of respiration of the food organism which is still alive (Mast, 1942) or from direct secretion of acid (Kitching, 1956b). The presence of a relatively large volume of indicator, acting as a buffer, could lead to errors in the method used by Mast. Digestion of Amoeba proteus is accompanied by a rise in pH to above neutral (Mast, 1942) as well as enzyme secretion. The rise in pH of pinocytosis vacuoles, occurring within an hour of ingestion (Nachmias and Marshall, 1961) is

parallel with the later phase of food vacuole development.

Early channels are said to be specifically related to mitochondria (Chapman-Andresen, 1963, Chapman-Andresen and Nilsson, 1960) following ingestion of sodium chloride and of sodium glutamate. The latter authors believe that micropinocytosis occurs from channels with the formation of small round cytoplasmic vesicles. The small vesicles observed may be the same as alpha-particles or the small round vesicles of this study (sections I and II). Mitochondria are present though relatively rare near alcian blue channels and other cytoplasmic organelles are absent from the area. If the section thickness is borne in mind, it can be seen that without extensive serial sections, a poor idea of mitochondrial frequency may be obtained. No suggestion of micropinocytosis has been seen at the peak development of alcian blue channels.

Mitochondria are much more common near sodium chloride induced channels and are so frequently involved in the sequestration phenomenon that they must lie close to rapidly developing channels. It is possible that they are more common near salt-induced channels where ion exchange may be occurring but the stage of rapidly developing and finely divided channels is poorly analysed after alcian blue. Small round droplets possibly resulting from micropinocytosis are found round fusion vacuoles after

sodium chloride and PTA staining vesicles, probably of the same origin are found near sodium chloride channels (microg.84). Channel fragments and droplets fuse to form complexes. Evidence for coalescence is found in other work (Chapman-Andresen and Holtzer,1960) and in general is characteristic of amoeba physiology (Andresen,1956). There is no evidence here that other cytoplasmic constituents are involved in complexes except as a result of sequestration. Ingested material has never been found in contractile vacuoles or the small satellite vesicles in the way that finely dispersed colloids may be (Brandt and Pappas,1962) possibly because the inducers used are not separated from the filamentous material and the ABM globules are too coarse.

The small dense bodies found on normal plasmalemma tend to accumulate on fusion droplets. Although their significance is doubtful, it may well suggest loss of membrane without loss of dense bodies. The dense bodies are not present on the small round vesicles and if these are produced by micropinocytosis the dense bodies would accumulate on the large vacuoles.

Consideration of fusion of two vacuoles shows that unless an increase in total volume or net loss of membrane occurs, an excess of membrane must build up. If the volume remains constant, the fusion vacuole has a smaller surface area than its precursors. The problem recurs in

consideration of contractile vacuole physiology (section II). Basically it is not known how quickly membrane can be broken down or synthesised in Amoeba proteus.

The type of fusion droplet in micrograph 80 must be the result of a fusion process. Membrane is incorporated into the lumen of the vacuole. A further degree of membrane incorporation is found in micrograph 81 with accumulation of membrane and ingested material. During this time the membrane still looks like plasmalemma and stains with PTA.

Closer examination of the fusion droplets shows that by applying a modification of the method employed in section II to estimate the relative volume occupied by mitochondrial tubules (Loud, 1962) it is possible to obtain a very approximate estimate of the quantity of membrane present in such a fusion vacuole. The figures obtained show some interesting facts. If we assume the precursors of the droplet to be channel fragments like that in micrograph 76 used in the assessment in appendix 6, the calculations show that about 13 channels would be needed to provide the volume but only 5 to provide the membrane. The discrepancy may be the result of one or more of several factors:-

1. membrane is probably actively destroyed
2. large fusion droplets with relatively little membrane (e.g. micrograph 78) may be incorporated to increase the volume with less increase in membrane.
3. secretion of enzymes may increase the volume
4. less complex channels would have a smaller surface area for the same volume.

However it is interesting to note that it is not essential to postulate dehydration to explain the appearance of such fusion droplets.

In another protozoan (Carchesium) the presence of lactose in the outer medium, a substance to which plasmalemma is normally impermeable, prevents the rapid initial shrinkage of newly ingested food vacuoles. Since the lactose cannot cross plasma membrane it exerts an osmotic effect on the cytoplasm. The balancing of the initial shrinkage must be due to this effect and hence shows that food vacuole walls initially retain their impermeability to lactose. After some minutes, however, the volume of the vacuole suddenly decreases. Kitching (1956b) in describing these experiments suggests that a sudden change in the permeability characteristics of the membrane occurs at this time. A similar change in permeability is postulated by Chapman-Andresen and Holter (1956) to explain the fact that ^{14}C labelled glucose which does

not penetrate intact amoeba plasma membrane becomes available to the cytoplasm when incorporated in pinocytosis vacuoles. In ^{14}C labelled glucose solution, pinocytosis does not occur and radio-active carbon is not metabolised. When a protein inducer is added, pinocytosis occurs, the cytoplasm becomes radio-active and $^{14}\text{CO}_2$ is released. Roth (1960) believes the explanation lies in the vast increase in surface area which allows a normally unmeasurable degree of diffusion to become significant. Kitching's (1956b) findings in Carchesium lend support to a qualitative change in the membrane.

The outermost membrane of all pinocytosis vacuoles and channels remains intact in this work, in contrast to Bennett's hypothesis (1956) which requires the dissolution of membrane to release ingested material into the cytoplasm.

The complex droplets take fusion a stage further to involve the destruction of membrane. Plasma membrane consists of protein and phospholipid (Engström and Finean, 1958) and the peripheral lamellated bodies of the droplets suggest there may be free phospholipid or lipoprotein present in a less organised form. There are similar findings in the food vacuoles which lend support to the relatively low capacity of Amoeba proteus in phospholipid metabolism. This is suggested on other grounds by Chapman-Andresen and Prescott (1956) and Wolpert and O'Neill

(1962) and may prove to be a limiting factor in pinocytosis or phagocytosis in other cells (Karnovsky and Wallach, 1961).

In order to explain the digestion of membrane there must be secretion of enzymes into the pinocytosis vacuole. Proteolytic enzymes occur in amoebae (Holter and Løvtrup, 1949) and are found in the heavier pole of Pelomyxa after centrifugation. They may be associated with the food vacuoles in the region. Hydrolytic enzymes also occur (Holter, 1955, Holter and Lowy, 1959) and acid phosphatase is thought to be associated with pinocytosis vacuoles (Birns, 1960). There is no evidence for large scale protein synthesis accompanying pinocytosis, as would be provided for example by a close association between cytomembranes and vacuoles or channels. It is usually considered that satellite vesicles of food vacuoles represent micropinocytosis (Roth, 1960) but they could be fusing with the vacuoles rather than separating from them (section II). It is then possible that these small round vesicles fuse with the food vacuoles or pinocytosis vacuoles and in doing so introduce enzymes. It is of interest in this respect that in centrifuged amoebae Holter (1955) showed acid phosphatase in the mitochondrial region but not in the mitochondria. Examination of this region shows that small round vesicles as well as mitochondria are

present. However, considerations of diameters show that the free vesicles are not the same as the food vacuole satellites even though they could be functionally related. The findings in the Golgi apparatus show that small vesicles probably originate from Golgi membranes and that these vesicles do not differ significantly from the food vacuole satellites. It is conceivable, therefore, that small vesicles produced in the Golgi apparatus contain enzymes and fuse with the food vacuoles. Further consideration is pure speculation.

Rose (1957) described small bodies or microkinetospheres which fuse with pinocytosis droplets in HeLa cells.

It is extremely common to find lamellated masses or fragments in dense cytoplasmic bodies of many cells within which particles ingested by pinocytosis or phagocytosis are found (e.g. Miller, 1961, Hayward, 1961, Karrer, 1960c, Walker, 1960). Such bodies may well be the same as lysosomes (Novikoff, 1961b) containing a battery of hydrolytic enzymes including acid phosphatase (de Duve, 1960). The peribiliary bodies of liver segregate injected bile pigments (Essner and Novikoff, 1960) and also contain acid phosphatase (Holt and Hicks, 1961, Essner and Novikoff, 1961) as well as esterases (Holt, 1956). Food vacuoles and small peripheral vesicles, possibly spontaneous pinocytosis

vacuoles in Pelomyxa carolinensis contain acid phosphatase (Birns, 1960) and the enzyme increases in quantity in feeding amoebae (Andresen and Holt, cited by Holt and Hicks, 1961). Enzymes do, therefore, occur in structures similar to the complex vacuoles and it is credible that they may be present in those found after alcian blue pinocytosis for the sole purpose of dealing with the plasmalemma since no other digestible substance is present. Both sequestration and the new membrane formed round channels are probably outside normal amoeba physiology.

Mitochondria and other cell organelles have recently been found encircled by membranes in normal cells (Moe and Behncke, 1960) and poisoned (Hruban et al, 1962) or malignant cells (Svoboda, 1962). Gey et al (1955) claimed that pinocytosis vacuoles in cultured cells fused with mitochondria, and Rhodin (1954) believed protein ingestion bodies in kidneys involved mitochondria in the same way. Although there is evidence against this in kidney (Niemi and Pearse, 1960) recent reports have again suggested mitochondria may be incorporated in certain situations (Novikoff and Essner, 1962).

It seems unlikely that sequestration is directly related to these phenomena, but that it is an incidental process with no general applications.

The deterioration of the mitochondria when dissociated from its normal environment is of interest. Palade (1956) suggested normal movement through the cell was necessary for mitochondria to contact the substrates for oxidative phosphorylation and those mitochondria which were fixed had important relations to membranes or "areas of intensive activity". Amoeba mitochondria may be particularly vulnerable in this respect because of the rapid rate of cytoplasmic mixing in normal conditions (see section II).

The segregation of channel fragments by newly formed membranes possibly from cytomembranes, seems to be unprecedented and probably occurs in response to extreme conditions or even as a terminal phenomenon. A study of later development is required.

The general problem of pinocytosis. Since Palade (1953) invoked pinocytosis to explain the presence of small cytoplasmic vesicles in capillary endothelium there has been a tendency to regard all intracellular vesicles in this light.

Cine micrographs from cultured cells, mentioned earlier, give evidence of microscopic droplet ingestion. Convincing evidence of fluid ingestion on a submicroscopic scale has been obtained by introducing electron dense markers, usually of a colloidal nature, into the extracellular

fluid. By this means a wide variety of tissue cells, among them the endothelium of arteries (Buck,1958), peritoneal mesothelium (Odor,1956), and epithelium of suckling small intestine (Clark,1959), renal glomeruli (Farquhar, Wissig, and Palade,1960) and tubules (Miller, 1962), gall-bladder (Hayward,1962b) and urinary bladder (Walker,1960) are shown to ingest material in discrete droplets. In many cases normal cell constituents are found to be involved in the process and it is reasonable to assume that it is part of normal cell physiology. However, the part played is not always clear.

An example of pinocytosis for discussion can be taken from the gall-bladder epithelium. There is evidence from normal micrographs that the brush border of the epithelial cells takes part in pinocytotic activity (Yamada,1955, Hayward,1962a). It has been shown that colloidal materials are indeed ingested in such a way that they enter apical droplets and eventually occur within dense cytoplasmic granules which are normally present (Hayward,1962b). Such granules contain membrane fragments and probably result from fusion and dehydration of ingested vesicles. Their morphological resemblance to the peribiliary bodies of liver raises the suspicion that they may be lysosomes (de Duve,1960). It will be necessary to demonstrate acid phosphatase or some other hydrolytic

enzyme to prove their identity. It is of interest that large pale droplets found in gall-bladder (Hayward, 1962a) are very similar to the protein absorption droplets of kidney (Novikoff, 1961b). They contain extensive myelin figures. Esterases occur in this same region of gall-bladder cells (Yamada, 1962) and Holt (1956) has shown esterases to be present in kidney droplets and in liver peribiliary bodies.

The evidence associating pinocytosis and such bodies is becoming stronger (Novikoff, 1961b).

Gall-bladder epithelium functions by the absorption of water and salts, probably by a neutral sodium chloride pump (Diamond, 1962). At best unmodified pinocytosis resorbs whole bile. If adsorption occurs (as in amoebae) a concentrated fraction would be taken in and dilution (on a very small scale) would result.

At the present time it is not easy to see any useful physiological similarities between the amoeba and metazoan cells undergoing pinocytosis. The biggest hiatus is the apparent absence of inducers in the latter. Only the demonstration of insulin activity by Barnett and Ball (1960) (see introduction) and Paul and Pearson's (1960) work on insulin in tissue cultures have come near to filling this gap. One might however seek control of pinocytotic activity by suppressors rather than inducers. Cortisone

suppresses pinocytotic activity in new-born small intestine (Clark,1959). Unfortunately except for the part played by general metabolic inhibitors (Schumaker, 1958) no specific inhibition of pinocytosis has yet been discovered in amoebae.

The second major difference appears to be in the ease with which amoebae dispose of extraneous ingested material by defaecation. Although pinocytosed substances can be expelled into inter-cellular spaces in the small intestine (Barnett,1959), there is a tendency for ingested material to accumulate in cells.

The most promising line of convergence of the phenomena in amoebae and metazoan cells is in the relationship to hydrolytic enzymes. It has been pointed out that pinocytosis and lysosomes have many connecting features (Novikoff,1961b) and it has been mentioned that food vacuoles in amoebae are related to lysosomal enzymes. Quertier and Brachet (1959) found that pinocytosed material is closely related to acid phosphatase activity and they and Holter (1955) believe amoebae may possess lysosome-like particles. It remains now to be shown which structure can fill this role and what part it plays in the response to pinocytosis induction.

APPENDICES 1 - 6

GRAPHS 1 - 6

APPENDIX 1

The Nomenclature of the species used

Amoeba proteus may have been figured by Rösse von Rosenhoff in 1755 under the name "der kleine Protheus" and Schaeffer (1917) gives an impressive list of synonyms given to organisms which were probably identical. Mast and Johnson (1931) maintain that Rösse's drawings were of a totally different organism (of Mycetozoa) and despite evidence to the contrary (e.g. Taylor, 1932) it seems unnecessary to take this early publication into consideration. Most authors now follow the description of Leidy (1878) and refer to the organism as Amoeba proteus.

The problem in the case of the giant amoeba is more fundamental since it hinges round the generic identification of the organism. Wilson (1900) described it and largely because of size included it in the same genus as the well known Pelomyxa palustris. There is no doubt on morphological grounds (Schaeffer, 1926; Andresen, 1956) that the giant amoeba is closely related to Amoeba proteus. Andresen (1956) firmly maintains that high power light microscopic fields of the two amoebae are indistinguishable and he would place both in a single genus Chaos, with its origin in Linnaeus' Systema Naturae, as Chaos chaos and Chaos diffluens (syn. A. proteus). The

name Amoeba proteus is too firmly established for this to be followed. The two species differ in size and number of nuclei and some authors feel this has generic significance (Wilson, 1900, Rice, 1945, Wilber, 1945, Kudo, 1946). However, Short (1946) finds that the diagnostic features of Pelomyxa palustris make it generically distinct and King and Jahn (1948) place all three organisms in separate genera (Amoeba; Pelomyxa; Chaos). The latter solution is probably the best and has effectively been accepted by some workers particularly of the Carlsberg school.

The name Pelomyxa carolinensis Wilson has been used in this work since, providing it is clear which species is employed the justification seems an academic exercise.

APPENDIX 2

Formulae of solutions used

1. Pringsheim's solution (modified after Chapman-Andresen, 1958)

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 20 mgm. per litre

KCl 26 mgm per litre

$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 200 mgm per litre

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 20 mgm per litre

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 2 mgm per litre

Total salt molarity = 1.40×10^{-3} (Chapman-Andresen, 1963).

Final pH about 6.6 (depending on that of the distilled water).

2. Fixatives

a. Palade's buffered OsO_4 (Palade, 1952).

Stock osmium tetroxide solution	2%
Sodium veronal	14.7 Gm.
Sodium acetate	9.7 Gm.
Distilled water to	500 ml.

The fixative is prepared by taking

5 ml buffer
 5 ml 0.1N HCl.
 2.5 ml distilled water
 12.5 ml. stock OsO_4 solution

The final pH is controlled to approximately 7.4 by adding acid or stock buffer.

b. Potassium permanganate (Luft, 1956)

Stock solution 1.6% KMnO_4

Equal parts of this stock and Palade buffer previously adjusted to pH 7.4 - 7.6 are mixed and used at once. A fall in pH results in precipitation. Precautions must be taken against the films of contamination which occur at the atmospheric surface. Fixation is at as near 0°C as possible.

3. Staining solutions

a. Karnovsky's alkaline lead solution.

Excess lead oxide PbO is added to about 25 ml boiling N/10 sodium hydroxide solution and boiling continued for 15 minutes. The solution is cooled completely and filtered. The resulting solution contains sodium plumbate and has a pH of 11 or more. It is used as 1:50 - 1:200 dilution in distilled water.

b. Phosphotungstic acid

10% aqueous solution.

c. Uranyl acetate solution - saturated solution in ethyl alcohol or rectified spirit.

APPENDIX 3

FIXATION METHODS

Strangeways and Canti (1927) showed that osmium tetroxide gave superior fixation of isolated cells examined in the light microscope. Baker (1958) criticises the use of the work to support the claims of OsO_4 as a fixative since the important properties of penetration and preservation through embedding procedures were not taken into account. Nevertheless osmium tetroxide has been used since the earliest attempts at biological electron microscopy. Results following methacrylate embedding, introduced by Newman, Borysko and Swerdlow (1949), proved to be non-uniform. Palade (1952) showed that, as it penetrates tissue, osmium tetroxide solution is preceded by a wave of acidification possibly as a result of its preferential reaction with basic amino-groups (Bahr, 1954). Palade therefore introduced a modified Michaelis buffered solution of osmium tetroxide which is the most popular fixative to-day.

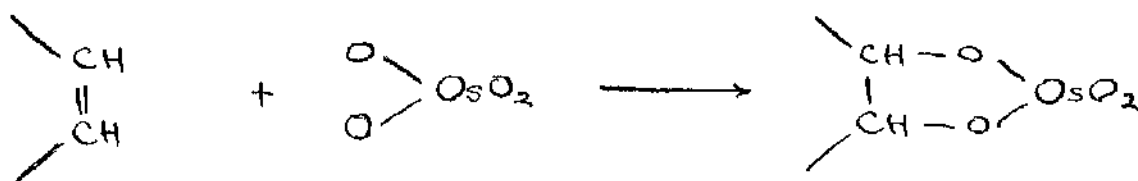
Malhotra (1962) has recently investigated the use of unbuffered solutions and has concluded that much of the damage in early work may have been due in part to the methacrylate embedding. Far more attention has been paid to the embedding material since the introduction of epoxy resins (see Glauret, 1963, for complete bibliography) which give superior preservation especially in highly

hydrated tissues such as cultured cells, protozoa, central nervous system and embryological tissues.

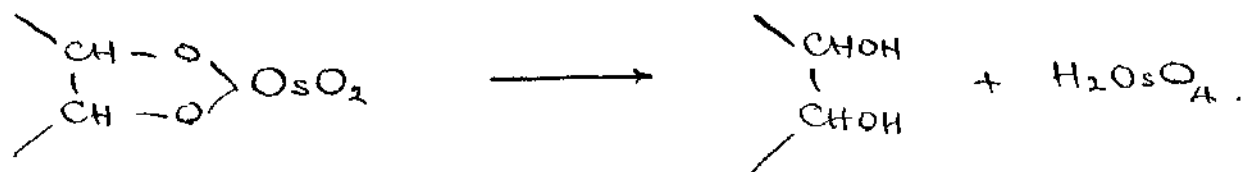
No attempt was made to try unbuffered solutions in this study.

Mechanism of OsO_4 fixation

Proteins: Baker (1958) suggests the reaction with proteins occurs at the double bond of tryptophane and histidine



with breakdown of the ring compound to a diol and a black osmium compound.



Bahr (1954) showed that the degree of reaction with proteins depends on the number of -SH groups present as well as the diamino acids and double bonds. Effectively therefore the cysteine, histidine and tryptophane content control the affinity for osmium. One drop of OsO_4 solution eliminates the nitroprusside reaction for -SH groups in a solution of cysteine. Amino groups react very strongly.

Lipids and hydrocarbons: Osmium tetroxide reacts with double bonds producing a characteristically brown compound. Bahr (1954) could show no reaction with fully saturated hydrocarbons. It is possible that osmium tetroxide dissolves in fat and is reduced by alcohols of dehydration (Baker, 1958) so that osmiophilia may not indicate true unsaturation.

The reaction of osmium tetroxide with lipids is important in the interpretation of fixed phospholipid-protein complexes, and the results are discussed in section III. Suffice it to say here that the initial interpretation (Stockenius, 1959) that the osmiophilic layer represents unsaturated hydrocarbon chains is no longer accepted. The electron dense substance lies at the site of the polarised hydrophilic ends of the molecules (Stockenius, Schulman and Prince, 1960) although it is still conceivable it could originate in the hydrocarbon chains and migrate to its final position (Finean, 1961).

Carbohydrates may react with OsO_4 without forming a precipitate (Pearse, 1960). A positive periodic acid Schiff reaction for glycogen can still be obtained after OsO_4 fixation (Karrer, 1960a, Lever, Jeacock and Young, 1961, Hayward, 1962a) possibly because fixation of associated protein reduces the solubility of the glycogen.

Potassium permanganate fixation

Osmium tetroxide and potassium permanganate are the only fixatives so far tried which preserve the spacing of myelin studied by X-ray diffraction, throughout preparation (Fineman, 1961).

Although known as a fixative since 1899 (Mönckeberg and Bethe) KMnO_4 was introduced into electron microscopy by Luft (1956). The mechanism of its action has received little attention. Important facts emerged from the study of Bradbury and Meek (1960). Gross swelling occurs on fixation in pure or mixed protein and phospholipid gels. Protein plugs "fixed" in KMnO_4 remain soluble in water until dehydrated in alcohol. Nuclei are rendered electron translucent although the Feulgen reaction remains positive (i.e. DNA is not extracted). The Sakaguchi reaction for arginine in basic proteins becomes negative so that the loss of nuclear substance is probably due to loss of nuclear basic protein. Tissues cease to be basophilic and cytoplasmic RNA particles disappear. The endoplasmic reticulum which is rich in phospholipid (section II) does not normally give a positive reaction with Baker's acid haematin but does so after KMnO_4 fixation. Bradbury and Meek believe the protein phospholipid complex to have been broken down.

Pineau (1961) shows that modifications produced in lipoprotein spacing in myelin by various experimental procedures designed to dehydrate the fresh tissue are visible in X-ray diffraction studies and after OsO_4 fixation but fail to appear in KMnO_4 fixed electron micrographs. He believes this may be due to some rehydration during fixation, possibly allied to the swelling noted above.

Plasma membranes and myelin, which is derived from Schwann cell membranes, show triple layering after osmium fixation but the inner most layer is sometimes poorly preserved and may be absent (Robertson, 1960). This probably indicates a chemical difference between the two dense layers of plasma membrane, substantiated in some cases by differential staining with phosphotungstic acid (Latta, 1962). Permanganate fixed myelin also shows a different thickness in the two layers but as fixation is prolonged the thinner intermediate layer becomes thicker until the two are indistinguishable in thickness. Permanganate is the fixative of choice to demonstrate unit membrane structures.

Recently Rosenbluth (1963) has shown differences between permanganate and osmium fixed spinal ganglia. After OsO_4 , cells commonly show serried vesicles "descending" from the plasma membrane. After permanganate, however, such

vesicles are replaced by two parallel membranes continuous with the plasma membrane. The suggestion is, therefore, that permanganate gives superior preservation of intracellular membranes which may tend to collapse into vesicles after OsO_4 .

Observations on permanganate fixed material

The principal differences observed have been in the nuclei, mitochondria and bacterial complexes.

In general the effect of permanganate fixation is of increased regularity of membranes as if by swelling or rounding off although the dimension of individual organelles are not increased. There is an accentuation of membrane and a loss of granulation in cytoplasm and on the cytomembranes. A structure consisting of concentric enclosed membranes is found in centrifuged Pelomyxa which is not recognisable from osmium fixed material (microg.15).

The nuclei (section II) fail to show a honeycomb structure, possibly because of a definite chemical difference between the two parts of the nuclear membrane. Following Bradbury and Meek, this could be due to a predominance of basic protein in membranes of the honeycomb itself which is likely to be of purely nuclear origin without the cytoplasmic affinities of the outer layer.

Mitochondrial tubules are decreased in number or even absent (microg.44). Each mitochondrion has limiting membranes of which the inner is less distinct than the

outer. Sometimes the outermost membrane is duplicated. Between the two limiting membranes circular profiles occur and sometimes this is the site of a myelin figure (microg.44). The pictures suggest that the outermost membrane of the mitochondrion remains intact but that the inner, together with its invaginations or tubules has collapsed into a single layer together with a few myelin figures representing phospholipid in its most stable form. The change is compatible, firstly with the established biochemical differences between the inner and outer membranes (Ball and Joel,1962) and secondly with a splitting of lipoproteins into proteins and free phospholipids postulated by Bradbury and Meek(1960).

The lamellated bodies of the inner vacuoles of the bacterial complexes show a variety of forms after osmium fixation which compare with the distortions of myelin produced by dehydration before fixation (Finean,1961). After permanganate however the picture is more constant with central foamy mass surrounded by concentric lamellae with marked triple layering and regular tubular structures. The tubules do not occur after OsO_4 . Since both Finean (1961) and Bradbury and Meek (1960) have shown the likelihood that permanganate fixation leads to swelling due to absorption of water the forms found here are believed to be due to the same mechanism i.e. to be artefacts of fixation.

APPENDIX 4 (i)The evaluation of small round vesicles of cytoplasm
of Amoeba proteus

A number of prints were selected and the diameter of the small vesicles of the cytoplasm measured. Only those with apparently sharp edges were measured, those obviously cut obliquely were ignored.

Vesicles in the following positions were measured:-

1. adjacent to the food vacuoles
2. adjacent to the contractile vacuoles
3. at the apex of the Golgi apparatus.

The vesicles of the cytoplasm were too sparse to obtain a large enough sample. Those near the Golgi apparatus were also very few (eight examples) but because of their theoretical interest they are included.

The populations obtained were treated as follows:-

1. For recording purposes they were regarded as normally distributed about a mean value; the arithmetic mean and the standard deviation calculated from the equations given and explained in Bernstein and Weatherall (1952).

2. because of the findings of Pelomyxa carolinensis that the distributions of diameters of small round vesicles of the mitochondrial region follow a curve with a negative skew rather than a normal distribution and that the

logarithmic distribution allowed some of the skew to be eliminated, the logarithmic distribution was calculated in each case and the probability of any two populations with such logarithmic distribution being the same calculated from 't' tables.

The following comparisons of populations were made:-

1. Between the Golgi vesicles and the food vacuole satellites.
2. Between the contractile vacuole microvesicles and the food vacuole satellites.
3. Between contractile vacuole satellites and Golgi vesicles.

General method

The formulae used were as follows, derived from Bernstein and Weatherall (1952):-

1. for the normal distribution

observations	=	x
Sum	=	$S(x)$
number of observations	=	n
arithmetic mean	=	$\frac{S(x)}{n} = \bar{x}$
standard deviation	=	$\sqrt{\frac{S(x - \bar{x})^2}{n-1}}$

2. for the logarithmic distribution

observations = x_1

(log) mean = $\frac{S(x_1)}{n} = \bar{x}_1$

ideal mean = a (i.e. usually the nearest whole number to \bar{x}_1)

then standard deviation = $\sqrt{\frac{S(x_1 - a)^2 - \frac{(S(x_1) - na)^2}{n}}{n - 1}}$

In order to calculate the functions shown, the following are tabulated diameter (x), log diameter (x_1), $(x - \bar{x})^2$, and $(x_1 - a)^2$.

Results1. diameter of food vacuole satellites (from prints numbers 193/5; 145/10; 132/5; 119/7; 123/2)

Diameter x	Log diameter x_1	$(x - \bar{x})^2$ 10	$(x_1 - a)^2$
2300 Å	3.362	20.25	0.026
1250	3.097	36.00	0.011
1040	3.022	65.61	0.032
1400	3.146	20.25	0.003
1400	3.146	20.25	0.003
1000	3.000	72.25	0.003
1400	3.146	20.25	0.040
1400	3.146	20.25	0.003
2000	3.301	2.25	0.003
3000	3.477	462.25	0.010
1300	3.114	30.25	0.077
1500	3.176	12.25	0.007
4500	3.653	702.20	0.204
2300	3.362	20.25	0.026
Sum	25820	1504.31	0.503

n = number of observations = 14

A. Calculations of normal distribution

$$\text{mean} = \frac{25820}{14} = 1830 \text{ \AA}$$

$$\text{Standard deviation} = \sqrt{\frac{1504.31 \times 10^4}{13}}$$

$$\text{i.e. mean} = 1830 \text{ \AA} \pm 1070 \text{ \AA}$$

B. for the logarithmic distribution of diameters of food vacuole satellites

$$\text{mean log.} = \frac{S(x_1)}{n} = 3.225$$

substitute ideal mean $a = 3.2$

$$S(x_1 - a) = 0.503$$

$$Sx_1 = 45.148$$

$$na = 44.8$$

$$\text{s.d.} = \sqrt{\frac{S(x_1 - a)^2 - \frac{(S(x_1) - na)^2}{n}}{n-1}}$$

$$= \sqrt{\frac{0.494}{13}}$$

$$= 0.195$$

i.e. (log) mean = 3.225 \pm s.d. 0.195.

2. The diameter of the satellite vesicles of contractile vacuoles (from prints numbers 187/8; 613/2; 176/2).

Diameter x	Log. diam. x_1	$(x - \bar{x})^2$ 4 10	$(x_1 - a)^2$
600	2.778	17.64	0.077
600	2.778	17.64	0.077
500	2.699	27.04	0.040
600	2.77	17.64	0.077
700	2.845	10.24	0.119
2500	3.998	219.0	0.806
1200	3.079	1.44	0.335
1500	3.176	23.04	0.457
1200	3.079	3.24	0.235
1800	3.255	60.84	0.570
1100	3.041	0.64	0.293
900	2.954	1.44	0.206
800	2.903	4.84	0.162
1400	3.146	14.44	0.417
600	2.778	17.64	0.077
400	2.602	38.44	0.104
600	2.778	17.64	0.077
1000	3.000	0.04	0.250
600	2.778	17.64	0.077
1800	3.255	60.84	0.576
800	2.903	4.84	0.162
1400	3.146	14.44	0.417
900	2.954	1.44	0.206
1300	3.114	7.84	0.377
Sums 25800	74.237	535.96	6.544

Calculation

- A. For the normal distribution of contractile vacuole satellites

$$Sx = 25800$$

$$n = 25$$

$$\text{mean} = \frac{25800}{25} = 1020 \text{ \AA}$$

$$\begin{aligned} \text{standard deviation} &= \sqrt{\frac{535.96 \times 10^4}{24}} \\ &= 960 \text{ \AA} \end{aligned}$$

$$\text{i.e. mean diameter} = 1020 \text{ \AA} \pm \text{s.d. } 960.$$

- B. For the logarithmic distribution of contractile vacuole satellites

$$S(x_1) = 74.237$$

$$\frac{S(x_1)}{n} = 2.969$$

$$a = 2.5$$

$$na = 62.5$$

$$\frac{(S(x_1) - na)^2}{n} = 5.505$$

$$\begin{aligned} \text{i.e. s.d.} &= \sqrt{\frac{6.544 - 5.505}{24}} \\ &= 0.208. \end{aligned}$$

$$\text{i.e. (log) mean} = \underline{\underline{2.969 \pm \text{s.d. } 0.208}}$$

Results

3. Golgi vesicle diameter, derived from specimen 139/4.

Diameter x	Log. diam. x_1	$(x - \bar{x})^2 \times 10^4$	$(x_1 - a)^2$
1500 Å	3.176	225	0.031
1200	3.079	2025	0.006
1600	3.204	25	0.042
1400	3.146	625	0.021
1400	3.146	625	0.021
1400	3.146	625	0.021
2200	3.342	3025	0.116
2500	3.398	7225	0.158
13200	25.637	14400×10^4	0.416

A. Normal distribution

$$n = 8$$

$$\sum(x) = 13200$$

$$\bar{x} = 1650 \text{ Å}$$

$$\sum(x - \bar{x})^2 = 14400 \times 10^4$$

$$\therefore \text{s.d.} = \sqrt{\frac{14400 \times 10^4}{7}}$$

$$= 450$$

$$\text{ie arithmetic mean} = \underline{\underline{1650 \text{ Å} \pm \text{s.d. } 450 \text{ Å}}}$$

B. Logarithmic distribution of Golgi vesicles.

$$S(x_1) = 25.637$$

$$\frac{S(x_1)}{n} = 3.204$$

$$\text{let } a = 3$$

$$\text{i.e. } na = 24$$

$$S(x_1) - na = 1.637$$

$$\frac{(S(x_1) - na)^2}{n} = 0.335$$

$$\therefore \frac{S(x_1 - a)^2 - (S(x_1) - na)^2}{n} = 0.081$$

$$\therefore \text{S.d.} = \sqrt{\frac{0.081}{7}}$$

$$= 0.1076$$

$$\text{i.e. (log mean)} = \underline{\underline{3.204 \pm \text{s.d. } 0.108.}}$$

Comparison of populations

General method (Bernstein and Weatherall, 1952).

Only the logarithmic distributions were compared.

for population a. $S(x_1^a - \bar{x}_1^a)$

for population b. $S(x_1^b - \bar{x}_1^b)$

for pooled measurement $S(x_1^a - \bar{x}_1^a) + S(x_1^b - \bar{x}_1^b)$

degrees of freedom $= n_a + n_b - 2,$

standard deviation

$$= \sqrt{\frac{S(x_1^a - \bar{x}_1^a) + S(x_1^b - \bar{x}_1^b)}{n_a + n_b - 2}}$$

= s.d.

standard error of each mean $= \frac{s.d.}{\sqrt{n_a}}$ and $\frac{s.d.}{\sqrt{n_b}}$

standard error of difference between the means

$$= \sqrt{\left[\frac{s.d.}{\sqrt{n_a}}\right]^2 + \left[\frac{s.d.}{\sqrt{n_b}}\right]^2}$$

and $t = \frac{\text{difference between means}}{\text{standard error of differences}}$

the corresponding value of P determined by seeking t and the degrees of freedom in published tables is the probability of the two populations being random selections

from the same population.

This is the same as the reciprocal of the odds against them being different. Since this covers the possibility of population a. being greater or smaller in size than b. and it can be seen by inspection if a. is bigger or smaller the actual probability is one half that from the tables (Bernstein and Weatherall, 1952). It is customarily taken that if P is less than 0.05 the populations differ significantly i.e. the odds against them being the same is greater than 20:1.

1. Comparison of food vacuole and contractile vacuole satellites.

Sum of deviations from mean squared $(\sum x_1 - \bar{x}_1)^2$

$$= \sum (x_1 - a)^2 - \frac{(\sum x_1 - na)^2}{n}$$

for food vacuoles = 0.494

for contractile vacuoles = 1.039

Sum = 1.533

degrees of freedom = total number of observations - 2

= 37

standard deviation = $\sqrt{\frac{1.533}{37}}$

= 0.203

∴ Standard error of means

for F.V. = $\frac{0.203}{\sqrt{14}}$

= 0.0544.

for C.V. = $\frac{0.203}{\sqrt{25}}$

= 0.0407.

Standard error of
difference

= $\sqrt{0.0544^2 + 0.0407^2}$
= 0.068.

$$t = \frac{3.225 - 2.969}{0.068} \quad (\text{difference of means})$$

$$= \frac{0.256}{0.068}$$

4

P = less than 0.01.

the populations are quite significantly different.

The contractile vacuole satellites and the food vacuole satellites differ significantly in their diameter.

2. Comparison between Golgi vesicles and food vacuole satellites.

Sums of (deviations from the mean) squared

$$= \sum (x_1 - a)^2 = \frac{(\sum x_1 - na)^2}{n}$$

for FVs = 0.494

for Golgi = 0.081

Sum = 0.575

degrees of freedom = 21

standard deviation = $\sqrt{\frac{0.575}{21}}$

= 0.1655

Standard errors of the means.

$$\text{for FVs.} \quad = \quad \frac{0.165}{\sqrt{14}} = 0.0442$$

$$\text{for Golgi} \quad = \quad \frac{0.165}{\sqrt{8}} = 0.0585$$

$$\text{Standard error of difference} = \sqrt{0.0442^2 + 0.0585^2}$$

$$= 0.0733$$

$$\therefore t = \frac{\text{difference between means}}{\text{standard error of difference}} = \frac{3.204 - 3.225}{0.0733}$$

$$= \frac{0.021}{0.073}$$

$$= 0.287$$

$$\therefore P = 0.75.$$

$$\text{and } \frac{P}{2} = 0.38.$$

\therefore these two populations are not significantly different
The food vacuole satellites and Golgi vesicles do not
differ significantly in diameter.

APPENDIX 4 (11)

The small round vesicles of centrifuged Pelomyxa

Small round vesicles occur throughout regions 4 - 6a and diameters of sample populations were measured from a number of micrographs. The following regions were examined:-

1. Region 5. the main mitochondrial band
(e.g. microg.8).

2. Region 5 - 6a the more centrifugal part of the mitochondrial band where mitochondria and small vesicles are sparsely distributed in granular cytoplasm. No food vacuoles are present (e.g. microg.9).

3. Food vacuole region (6a) (e.g. microg.11).

The same method of measurement was adopted as with Amoeba proteus (Appendix 4(1)).

The samples were examined by constructing histograms to give an idea of the frequency distribution on a quantal basis.

Graph 1 shows such an arrangement for region 5. It is obviously not a normal distribution but has a pronounced negative skew. The arithmetic mean will therefore give a false low value and standard deviation an unreliable impression of scatter. There appear to be at least two explanations contributing to this shape of

curve. If diameters are plotted when in fact the volumes are normally distributed the curve will have a negative skew (Bernstein and Weatherall, 1952). This would be eliminated by plotting either (diameter)³ (Graph 2) or log.diameter (graph 3). It can be seen neither of these is totally effective although graph 3 is probably nearer a normal distribution.

The method of measurement cannot be reliable since the section thickness (about 1,000 Å) approaches the vesicle diameter. A large number will be cut so that their maximum diameter is measured but those cut slightly tangentially while not with obviously indistinct edges will give a falsely small diameter. There will be no balancing false large diameters and the distribution curve will be weighted to the left. This cannot be eliminated. A final alternative is a growing population reaching a certain size being destroyed.

Because of this finding the statistical analysis in appendix 4 (i) is based on a logarithmic distribution, particularly as the latter gives more realistic results where the standard deviation approaches the mean value (Bernstein and Weatherall, 1952). Because of the negative skew arithmetical means and standard deviations are not used here for recording purposes. The population is based on the maximum in the histogram. Thus most of the vesicles

of the mitochondrial band lie between 1,800 and 2,000 Å diameter.

The second sample from region 5 - 6a is represented on graph 5. It shows two maxima probably from two separate populations. The smaller and more frequent has a maximum between 1,400 and 1,800 Å. The larger and less common between 2,600 and 2,800 Å extending upwards. It is not possible to subdivide the sample in a satisfactory way.

Graph 4 shows the small vesicles of the food vacuole region. Once more they fall into two groups. The smaller with a maximum between 2,000 and 2,500 Å and the larger between 3,500 and 4,000 Å. Again it is not possible to subdivide the vesicles satisfactorily.

Thus we have the picture of a group of small vesicles extending from region 5 into the heavier parts:-

Region 5	1800 - 2000 Å	
Region 5 - 6a	1400 - 1800 Å	2600 - 2800 Å
Region 6a	2000 - 2500 Å	3500 - 4000 Å

and possibly decreasing in size with increased density and mixing with a second group of larger vesicles as the food vacuole region is approached. The figures obtained are inadequate to give an exact picture of the distribution and the figures are confusing in the absence of some additional factors, for example the histochemical

properties. It can be tentatively suggested that the smaller group correspond to those normally found free in the cytoplasm and the larger to the food vacuole satellites.

Comparison of small vesicles of mitochondrial band (region 5) and contractile vacuole satellites.

Mitochondrial band (region 5). (prints number 188/2 188/10).

diameter Å x	log. diam. x_1	$x^2 (x \cdot 10^4)$	$(x_1 - a)^2$
1500	3.176	225	0.0310
1400	3.146	196	0.0213
800	2.903	64	0.0094
1900	3.279	361	0.0778
1900	3.279	361	0.0778
1400	3.146	196	0.0213
1900	3.279	361	0.0778
1900	3.279	361	0.0778
1900	3.279	361	0.0778
1500	3.176	225	0.0310
1300	3.097	156	0.0094
1900	3.279	361	0.0778
2000	3.301	400	0.0906
1900	3.279	361	0.0778
3400	3.532	1156	0.2830
1500	3.176	225	0.0310
1500	3.176	225	0.0310
1900	3.279	361	0.0778
1500	3.176	225	0.0310
1900	3.279	361	0.0778
$S(x) = 34900$ $S(x_1) = 64.516$ $S(x^2) = 6542 \cdot x \cdot 10^4$ 1.2902			
$n = 20$ $a = 3$			

For the following sets of standard deviations,
the values for normal frequency are derived from:-

$$\text{mean} = \frac{\sum x}{n}$$

$$\text{s.d.} = \sqrt{\frac{S(x^2) - \frac{S^2(x)}{n}}{n-1}}$$

and for logarithmic distribution from:-

$$\text{mean} = \frac{S(x_1)}{n}$$

$$\text{s.d.} = \sqrt{\frac{S(x_1 - a)^2 - \frac{(S(x_1) - na)^2}{n}}{n-1}}$$

where a is the nearest whole number to the mean value
of x_1

Normal frequency distribution of diameters - for
reasons why this is inaccurate see the earlier discussion.

(region 5) arithmetic mean = $\frac{34900}{20} = 1750 \text{ \AA}$

and, from above s.d. = $\sqrt{\frac{6542 \times 10^4 - 6040 \times 10^4}{19}}$

$$= 514 \text{ \AA}$$

\therefore arithmetic mean = $1750 \text{ \AA} \pm 514 \text{ \AA}$

Logarithmic frequency distribution of region 5 vesicles.

$$\text{mean} = \frac{64.516}{20} = 3.2258$$

$$\text{s.d.} = \underline{0.0607}$$

$$\log \text{ mean} = \underline{3.2258 \pm \text{s.d. } 0.0601}$$

Diameter of contractile vacuole satellite vesicles. (from prints number 187/4; 188/4).

Diameter Å x	Log. diam. x_1	$x^2 (\times 10^4)$	$(x_1 - a)^2$
1400	3.146	196	0.0213
1500	3.176	225	0.0309
2050	3.312	420	0.0973
1900	3.279	361	0.0778
1250	3.097	156	0.0094
1250	3.097	156	0.0094
1900	3.279	361	0.0778
1900	3.279	361	0.0778
1900	3.279	361	0.0778
1450	3.161	210	0.0259
1090	3.033	118	0.0109
1700	3.230	289	0.0529
1250	3.097	156	0.0094
1090	3.037	118	0.0137
1250	3.097	156	0.0094
1250	3.097	156	0.0094
1350	3.130	182	0.0169
1350	3.130	182	0.0169
2090	3.320	436	0.1024
1250	3.097	156	0.0094
30170	69.373	4756×10^4	0.7657

$$a = 3$$

$$n = 20.$$

For normal frequency distribution

$$\text{arithmetic mean} = \frac{30170}{20}$$

$$= \underline{1508 \text{ Å}}$$

$$\text{and, from formulae above, s.d.} = \underline{330 \text{ Å}}$$

$$\text{i.e. mean} = \underline{\underline{1508 \text{ Å} \pm 330}}$$

B. For logarithmic distribution

$$\log \text{ mean} = \frac{63.373}{20}$$

$$= 3.168$$

$$\text{and, from formulae above, s.d.} = 0.102$$

Comparison of these two populations (for formulae see page 168, Appendix 4 (i)) using logarithmic distribution.

$$\log \text{ diameter} = x_1$$

$$\text{mean log diameter} = \bar{x}$$

for mitochondrial band (population 1).

$$S(x_1 - \bar{x})^2 = 0.0702 \quad \text{mean} = 3.226 \quad n = 20.$$

for contractile vacuoles (2)

$$S(x_1 - \bar{x})^2 = 0.1977 \quad \text{mean} = 3.168 \quad n = 20.$$

$$\text{Sum} = 0.2679 \quad \text{diff.} = 0.058 \quad N = 40.$$

$$\text{Degrees of freedom} = 38$$

$$\text{Standard deviation} = \sqrt{\frac{0.2679}{38}}$$

$$= 0.084.$$

$$\text{Standard error of mean for population 1} = \frac{0.084}{\sqrt{20}}$$

$$= 0.019.$$

$$\begin{aligned}\text{Standard error of mean for population 2} &= \frac{0.084}{\sqrt{20}} \\ &= 0.019\end{aligned}$$

$$\begin{aligned}\therefore \text{Standard error of difference} &= 0.019\sqrt{2} \\ &= 0.0267\end{aligned}$$

$$\begin{aligned}\therefore t &= \frac{0.058}{0.0267} \text{ (difference in means)} \\ &= 2.18\end{aligned}$$

$$\therefore P = \text{less than } 0.05$$

The two populations show no significant similarity in diameter.

APPENDIX 5Theoretical aspects of mitochondrial structure

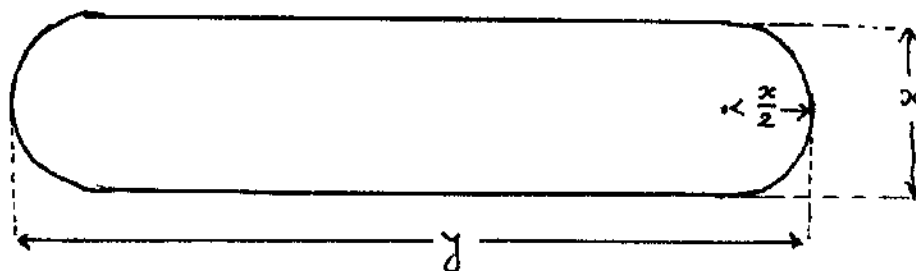
1. The relationship between surface area of tubules and total surface area of the mitochondrion (i.e. the surface area presented to the cytoplasm).

If we consider the ideal case of a mitochondrion

length y

width x

and assume that it is cylindrical and has hemispherical end surfaces, containing tubules of diameter d with a total effective length l .



The total surface area of this structure (i.e. SA_m)

$$= (y-x)\pi x + 4\frac{x^2}{4}$$

$$= \pi x y$$

and the total volume $= (y-x)\pi \frac{x^2}{4} + \frac{4}{3}\pi \frac{x^3}{8}$

$$\text{i.e. } V_m = \frac{\pi x^2}{4} \cdot (y - \frac{x}{3})$$

The corresponding volume of a cylinder of these dimensions would be $\pi \frac{x^2}{4} y$

and in order to simplify this ideal case the factor $\frac{x}{3}$

is ignored, and the volume taken to be $\pi \frac{x^2}{4} y$

The surface area of the tubules $= l \pi d$ (i.e. SA_c)

and the volume of the tubules $= l \pi \frac{d^2}{4}$ (i.e. V_c)

The relative surface area $= \frac{SA_c}{SA_m} = \frac{l \cdot \pi \cdot d}{\pi x y} = \frac{ld}{xy}$

the relative volume $= \frac{V_c}{V_m} = \frac{l \pi \frac{d^2}{4}}{\frac{4}{y \pi x^2}} = \frac{ld^2}{yx^2}$

therefore $\frac{\text{the relative surface area}}{\text{the relative volume}} = \frac{SA_c}{SA_m} \cdot \frac{V_m}{V_c}$
 $= \frac{ld}{xy} \cdot \frac{yx^2}{ld^2}$
 $= \frac{x}{d}.$

Similar calculations can be made about the cristaeform mitochondria of metazoan tissues.

If there are n complete cristae of thickness d running across the width of the mitochondrion x , then with the reservations mentioned above,

$$\begin{aligned} SA_m &= y \pi x \\ V_m &= y \pi \frac{x^2}{4} \\ SA_c &= n \cdot 2 \cdot \pi \frac{x^2}{4} \\ V_c &= n \cdot d \cdot \pi \frac{x^2}{4} \end{aligned}$$

$$\text{therefore } \frac{\text{relative SA}}{\text{relative volume}} = \frac{\frac{SA_c}{SA_m}}{\frac{V_c}{V_m}}$$

$$= \frac{x}{2a}$$

It can be seen that from these simple formulae that an increase in size (i.e. diameter) of the mitochondrion with the same size of tubules or cristae results in a greater surface area for the same relative volume. Larger tubules or thicker cristae result in a smaller surface area for the same relative volume but the decrease is greater in the case of the cristae. Increasing the number of cristae or tubules does not affect this ratio, though it does alter the absolute values.

Quantitative observations on mitochondria of Amoeba proteus

A grid of parallel lines spaced at 0.5 or 1.0 cm. was superimposed on each mitochondrion and the following measurements made:-

the length of each line directly superimposed on a mitochondrion. The sums of these lengths = S(lm)

the total length of each line directly superimposed on a tubule. The sum of such lengths = S(lt)

$$\text{then } \frac{S(lt)}{S(lm)} = \frac{Vt}{Vm} \quad (\text{Loud, 1962})$$

The grid was rotated through 90° and the

measurements repeated and the average of the two recorded.

The following table has been constructed from measurements and theoretical calculations for a small series of mitochondria.

$\frac{V_t}{V_m}$ represents V_R the ratio of tubule volume to

mitochondrial volume, x, y and d measured in mms , are the dimensions of each mitochondrion and its tubules from which $\frac{SA_R}{V_R}$ ($= \frac{x}{d}$) is calculated. The surface area of the tubules relative to the total area of the mitochondrion can then be calculated ($\frac{SA_R}{V_R} \times \frac{V_t}{V_m}$).

	Print	mag $\times 10^3$	$\frac{S(1t)}{S(1m)}$ $\frac{V_t}{V_m} \times 100$	x diam	y len	d tubule diam	$\frac{SA_R}{V_R} = \frac{x}{d}$	SA_R
1	673/23	56	20%	72	135	4.5	16	3
2	673/23	56	19.5%	70	160	4.5	16	3
3	562/13	37	30%	45	55	3.0	15	5
4	562/13	37	17%	60	60	3.0	20	3
5	510/13	56	13.8%	80	250	5.0	16	2
6	91/9	23	14%	27	52	1.5	18.4	3
7	92/2	?	20%	23	35	1.5	15.3	3
8	92/2	?	23%	26	36	1.5	17.3	4

In a bigger series of 52 mitochondria $\frac{X}{d}$ has been measured and is shown quantally in graph 6. There is an approximately normal distribution. The arithmetic mean is approximately 16 and from this large population probably approaches the average value for the whole.

The tabulated observations on a limited series show that volume ratio fluctuates widely between 13.8% and 30% and the ratio $\frac{X}{d}$ or $\frac{SA}{V_R}$ also fluctuates.

It cannot be expected that $\frac{X}{d}$ would be constant. Firstly it is a matter of simple observation that tubule diameter is more restricted than mitochondrial diameter and secondly the true diameter of a mitochondrion cannot be recorded since the plane of section may exaggerate it to an unknown degree. The ratio $\frac{X}{d}$ of 16 taken from graph 6 is therefore a crude estimate which may be greater than the true value.

The information from amoeba mitochondria can be compared with a tiny sample of vertebrate mitochondria. Those taken are as follows:-

1. epithelial cell of proventriculus of fowl
(courtesy of H.S. Johnston)
2. epithelium of colon of mouse (Hayward and Johnston, 1962)
3. spermatogonium of rat (Carasso and Favard, 1962)

4. cardiac muscle (courtesy of A.D. Hally).

The following figures were obtained:-

Type	$V_R\%$	$\frac{SA_R}{V_R}$ or $\frac{\pi}{2d}$	SA_R
1 Fowl ventriculus	32	30	10
2 Fowl ventriculus	30	27	9
3 Colon	14	8	1
4 Colon	17.6	8	1.5
5 Spermatogonia	30	9	3
6 Spermatogonia	40	30	12
7 Cardiac muscle	30	23	7
8 Cardiac muscle	16	16	2.5

Thus the volume ratio is of the same order and the surface area ratio varies widely from the same order as amoeba to a far higher level.

Total surface area of tubules

Andresen (1956) estimates the total volume of mitochondria of Pelomyxa carolinensis to be about 1.1% of the volume of the cell. He bases his calculations on a mitochondrial size of 1 by 1.5 μ , and amoeba volume of $5 \cdot 10^7 \mu^3$ and about 400,000 mitochondria per amoeba. From his observations it appears that the mitochondrial number is proportional to the volume of the cell.

If we assume that Amoeba proteus has a volume of about $\frac{1}{20}$ that of Pelomyxa and that the number of mitochondria remains proportional to volume, from

Andresen's figures, such an amoeba should contain approximately 20,000 mitochondria.

A mitochondrion with dimensions 2 by 1 μ has a surface area of $2\pi\mu^2$ and the total surface area of the mitochondria is $40,000\pi\mu^2$

Since $\frac{V_t}{V_m} = \frac{1}{5}$; and $\frac{SA_R}{V_R} = 16$ (from graph 6)

The ratio of surface area of tubules to surface area of mitochondria = $SA_R = 3.2$ (compare with the foregoing table) thus the total surface area of all the tubules

$$= 3.2 \times 10^4 \times 4\pi\mu^2$$

$$= \underline{\underline{\text{approx } 0.35 \text{ mm}^2}}$$

APPENDIX 6Consideration of the volume and surface area changes occurring during micropinocytosis

Assuming a large sphere diameter 10 microns

$$\begin{aligned}\text{then surface area} &= 4\pi r^2 \\ &= 4 \cdot \pi \cdot 25 \mu^2 \\ &= 100 \pi \mu^2\end{aligned}$$

$$\begin{aligned}\text{and volume} &= \frac{4}{3} \pi r^3 \\ &= \frac{500 \cdot \pi}{3} \mu^3\end{aligned}$$

giving rise to a small round vesicle diameter 0.4μ .

$$\begin{aligned}\text{surface} &= 4\pi r^2 \\ &= 0.16 \pi \mu^2 \\ \text{volume} &= \frac{4}{3} \pi r^3 \\ &= \frac{0.032 \pi}{3} \mu^3.\end{aligned}$$

Assuming no gain or loss of membrane other than by pinocytosis:—

$$\begin{aligned}\text{New surface area of food vacuole} &= (100 - 0.16) \pi \mu^2 \\ &= 4\pi r_1^2 \quad \left(\begin{array}{l} r_1 = \text{new} \\ \text{diameter} \end{array} \right) \\ \therefore r_1^2 &= \frac{100 - 0.16}{4} \\ \therefore r_1 &= \sqrt{25 - 0.04} \\ &= 4.9961.\end{aligned}$$

$$\begin{aligned}
 \text{new volume} &= \frac{4}{3} \pi r^3 \\
 &= \frac{4}{3} \pi (4.9961)^3 \mu^3 \\
 &= \frac{4}{3} \pi \cdot 124.7 \mu^3 \\
 &= \frac{498.8 \cdot \pi}{3} \mu^3
 \end{aligned}$$

$$\begin{aligned}
 \text{Decrease in volume} &= \left(\frac{500}{3} - \frac{498.8}{3} \right) \pi \mu^3 \\
 &= \frac{1.2 \cdot \pi}{3} \mu^3
 \end{aligned}$$

$$\text{Volume of small sphere} = \frac{0.032 \cdot \pi}{3} \mu^3$$

∴ The decrease in volume is 37.5 times as big as the loss of volume by pinocytosis.

Similarly the fusion of a small sphere with a large one without concomitant gain in volume produces an excess of membrane.

APPENDIX 6The concentration of membrane in a fusion body

If we consider the complex fusion body in micrograph 80 an estimate of the total volume enclosed by the outer membrane can be made by Loud's method (1962). A grid of parallel lines is superimposed on the micrograph and the total length of the lines crossing the body is measured. At the same time the total number of intersections between the lines and the membranes (of the inner mass and of the outer wall) is counted (table 1). Each is repeated in different grid positions.

	length of lines crossing body	intersections of membranes
1	1828	193
2	1826	184
3	1802	213
average	1815	197

It can be seen that the reproducibility of the method is not high but with three grids position covering 360° the average is taken.

Secondly considering the channel fragment shown in micrograph 76 we can repeat the procedures to estimate the volume and the number of intersections with the wall (or membrane).

The results are shown below

	length of line crossing channel	intersections of membrane
1	140	54
2	147	28
3	120	40
4	137	36
<hr/>		
total	544	158
average =	138	39

Once again the reproducibility of single measurements is poor but providing a number of grid positions are taken it is justifiable to take the mean value.

The absolute volumes of both channel and fusion droplet can be estimated from these figures by reference to a section through a standard sphere. However the ratio of the volume can be obtained by direct comparison between the two tables.

$$\text{i.e. } \frac{\text{vol. of fusion droplet}}{\text{vol. of channel}} = \frac{1815}{138} = \text{approx. } 13 \text{ times}$$

Similarly by taking average values (say t) for the width of a membrane intersection the absolute surface area or volume of membrane involved can be calculated.

But the ratio of membrane present can be estimated:-

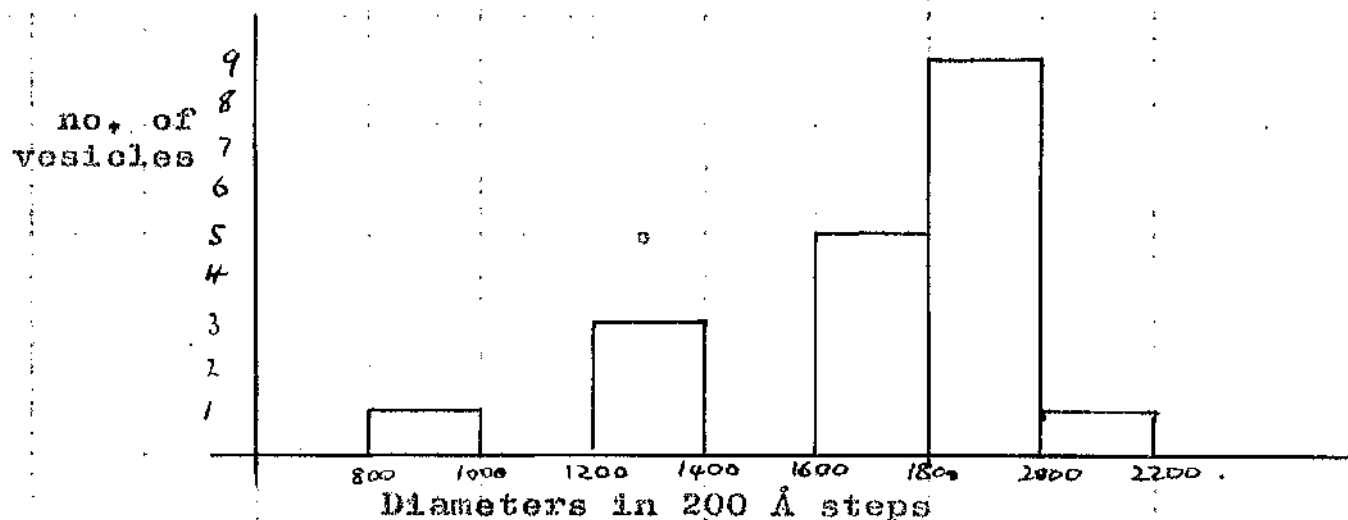
$$\frac{\text{membrane of fusion droplet}}{\text{membrane of channel}} = \frac{197}{39} \quad \underline{5 \text{ times}}$$

Thus it can be seen that whereas 13 channels of the type selected will fuse to produce such a complex, only 5 times the surface membrane is involved.

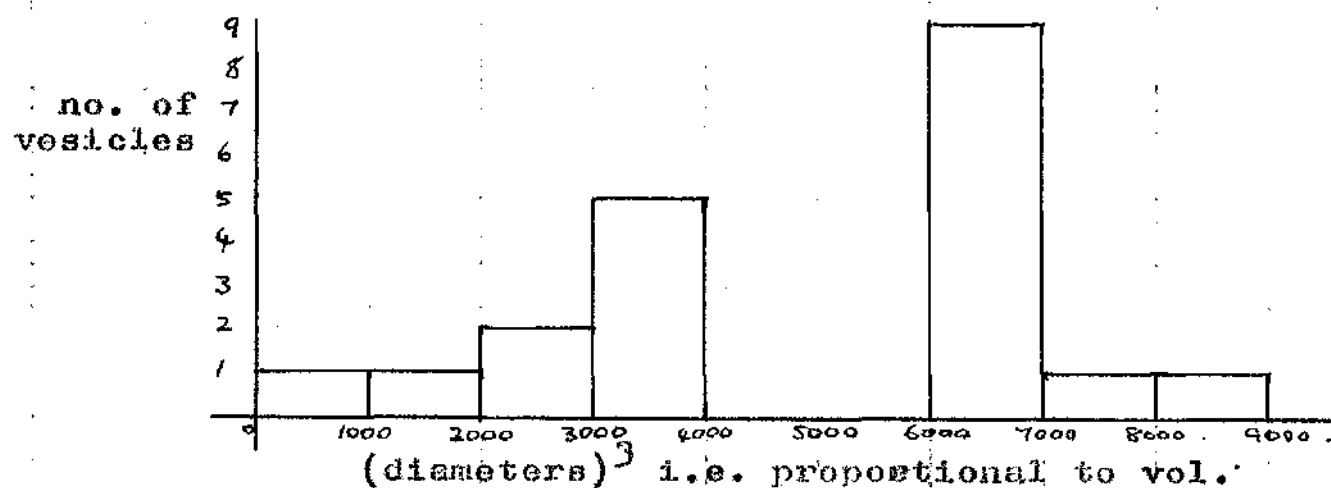
The mechanics of such figures has been discussed in section IV.

Graphs of the diameters of the small round vesicles of centrifuged Pelomyxa carolinensis arranged quantally

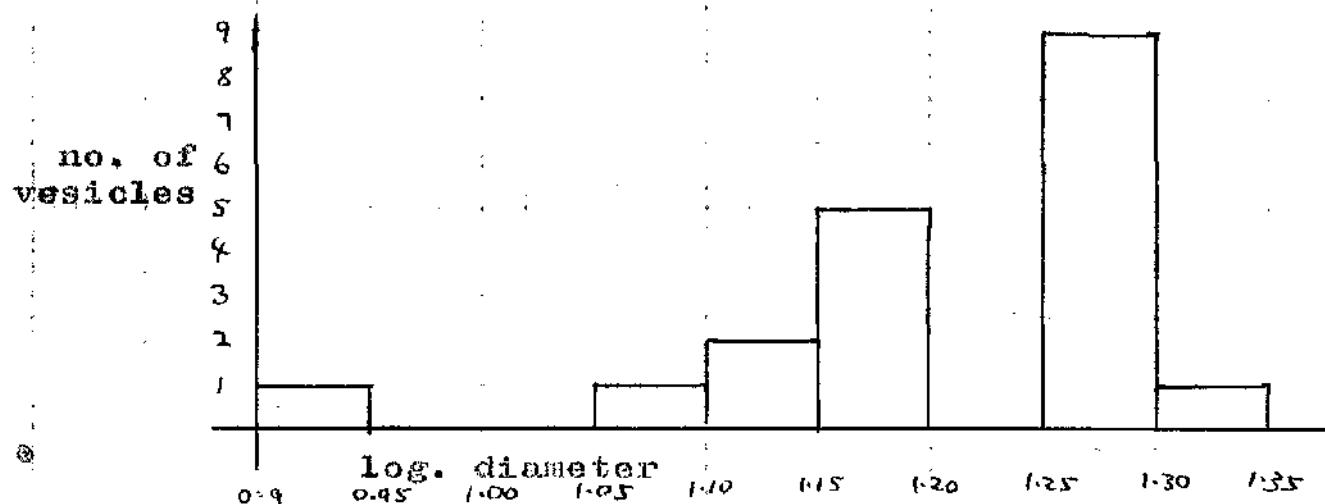
Graph 1. The main mitochondrial band

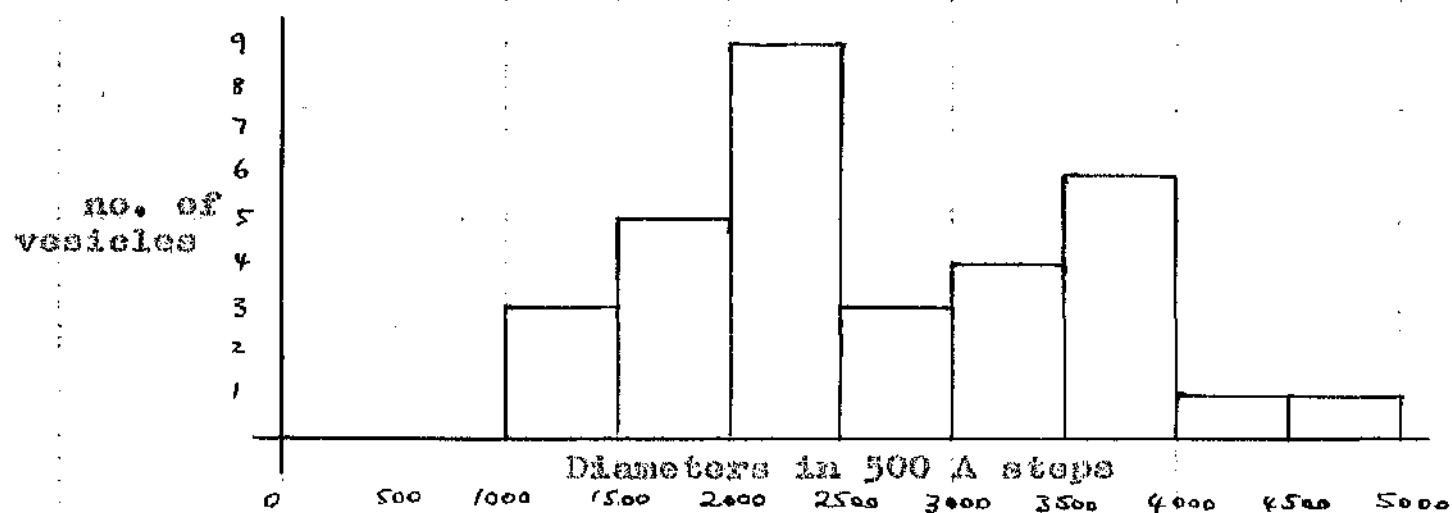
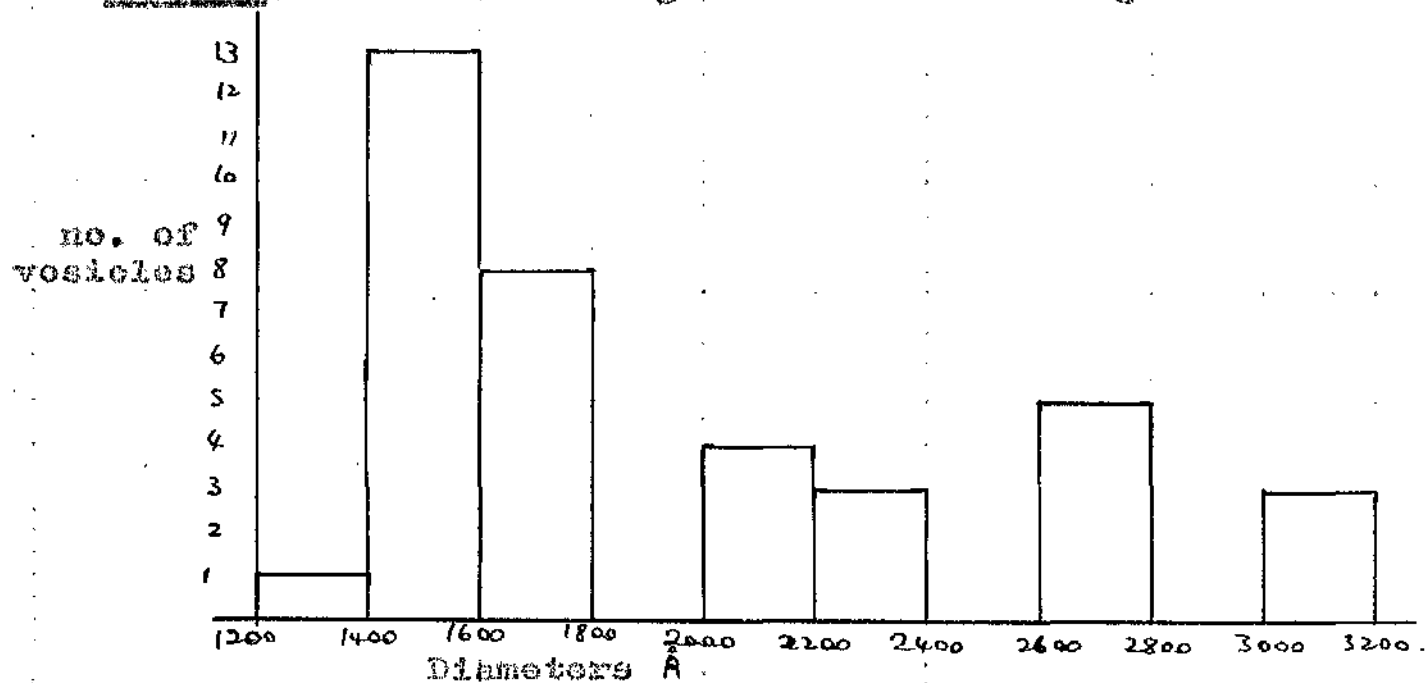


Graph 2. The same diameters cubed



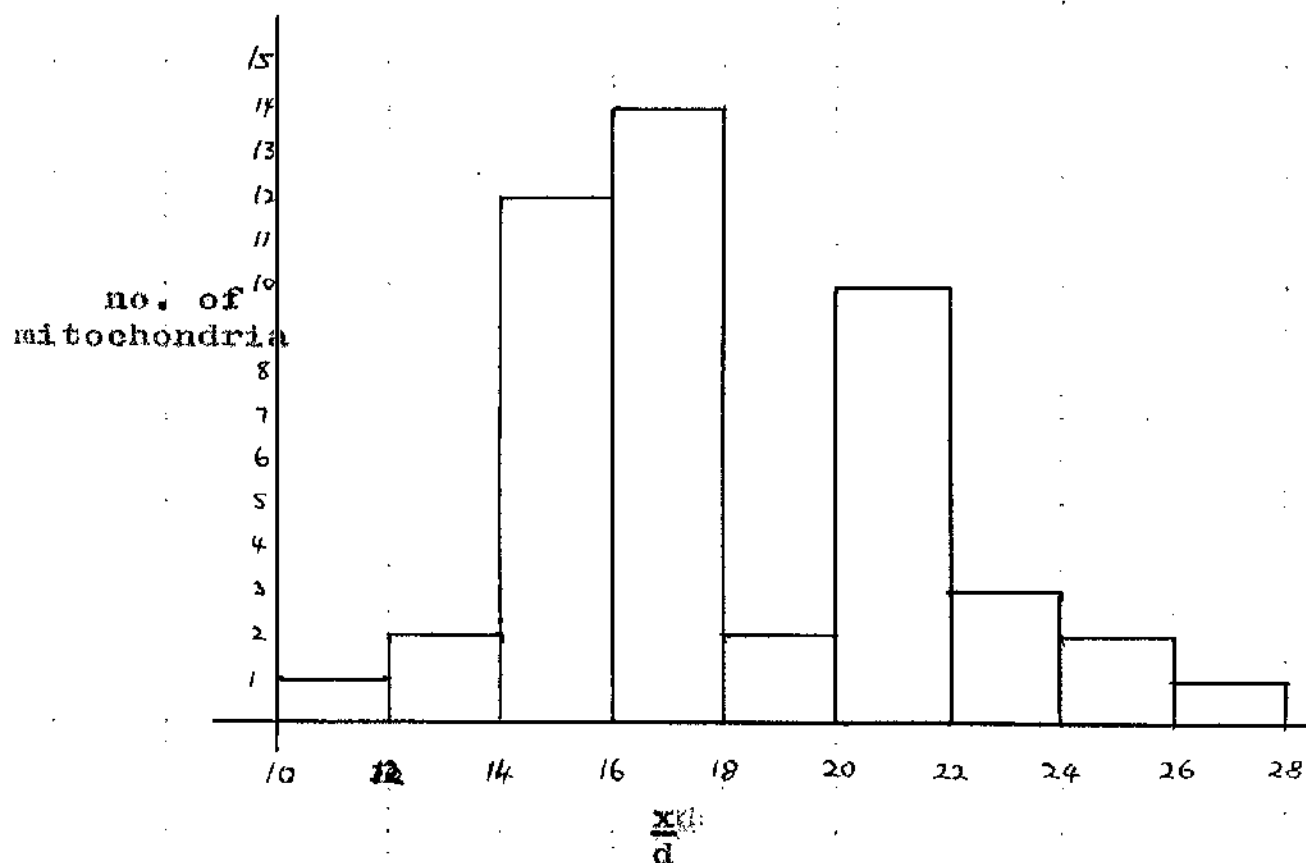
Graph 3. Logarithmic transformation



Graph 4. Food vacuole region.Graph 5. More centrifugal mitochondrial region

Graph 6

The distribution of the ratio $\frac{\text{relative surface area}}{\text{relative volume}}$ calculated theoretically for the tubular mitochondria of Amoeba proteus. In the ideal case the ratio is equal to $\frac{x}{d}$ and this has been measured for 52 mitochondria



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AN ELECTRON MICROSCOPIC STUDY OF AMOEBAE WITH PARTICULAR
REFERENCE TO INDUCED PINOCYTOSIS.

A.F. Hayward

Amoeba proteus was studied in thin sections by means of the electron microscope under normal conditions and after treatment with substances which induce pinocytosis - the ingestion of fluid by invagination of the plasma membrane to form cytoplasmic droplets.

The description of the nucleus, cytoplasmic membranes, mitochondria, fat droplets, crystals and Golgi apparatus confirms that already made by others and adds some details. In addition the relationship between volume and surface area of the mitochondrial membranes is examined. The structure of the heavy spherical bodies is described for the first time in the electron microscope. Each consists of a highly electron dense mass believed to contain mineral salts.

In support of these findings the closely related giant amoeba Pelomyxa carolinensis was examined after centrifugation in vivo. The structure of the strata produced is described in detail. Centrifugation confirms the fine structure of the heavy spherical bodies and further suggests that the small round cytoplasmic vesicles

found in both species represent the alpha particles of light microscopy.

The strain of Amoeba proteus used in the investigation is grossly infected with an unidentified bacterium which is accompanied by a specific lamellated structure probably of a lipid nature. The complex bacterial vacuoles are described and their general relationships to other structures is discussed.

Pinocytosis was induced with alcian blue and a few experiments were also carried out with sodium chloride and albumin.

The initial channel formation after alcian blue is accompanied by a definite hitherto undescribed change in cytoplasmic consistency. The cytoplasm becomes granular, and more firmly attached to the plasma membrane. The ingested membrane and the inducer are segregated into complex droplets and there is evidence that the membrane itself is digested. After very intense pinocytosis, the amoebae show signs of toxic effects. Mitochondria become grossly altered, ingested material becomes surrounded by an entirely new membranous structure possibly derived from the cytomembranes and masses of lamellated bodies probably representing phospholipid occur free in the cytoplasm. As a side effect of pinocytosis small fragments of cytoplasm become trapped

between two layers of membrane. The phenomenon is referred to as sequestration.

The findings in pinocytosis after sodium chloride and albumin induction are largely confirmatory.

The relationship between pinocytosis in amoebae and in other cells is briefly discussed.

AN ELECTRON MICROSCOPIC STUDY OF
AMOEBAE WITH PARTICULAR REFERENCE
TO INDUCED PINOCYTOSIS.

A.F. HAYWARD.

VOLUME 2

ILLUSTRATIONS

6 diagrams

103 micrographs.

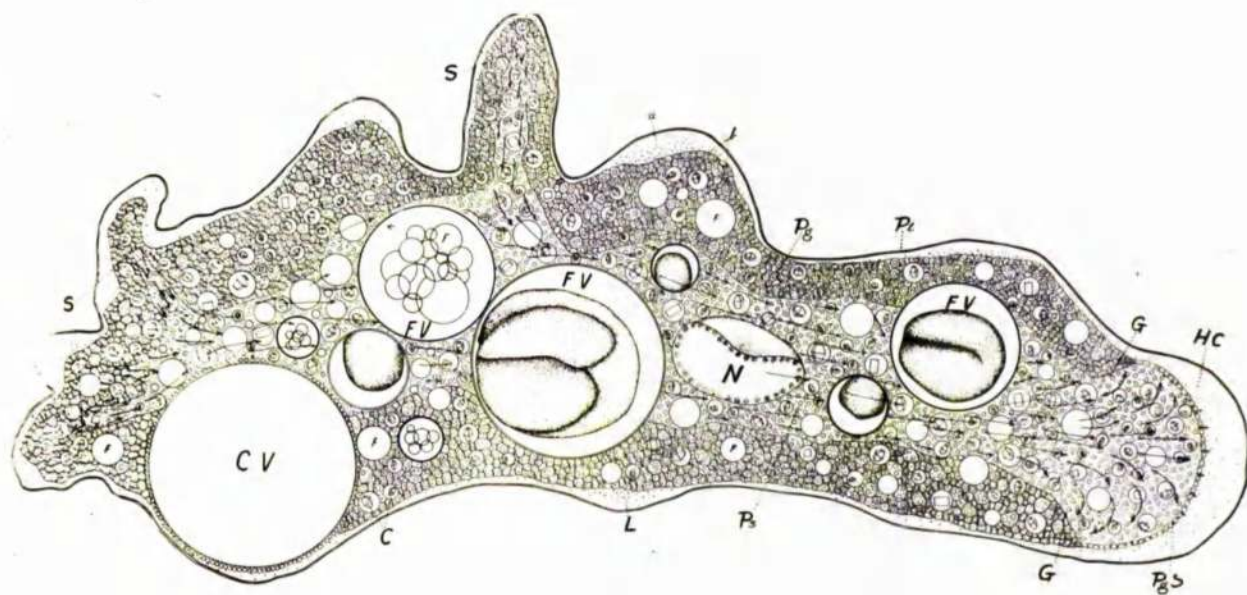
(Unless otherwise stated the specimens
are fixed in osmium tetroxide and
unstained).

Diagrams 1. Diagrammatic optical section of Amoeba proteus (reproduced from Mast 1926).

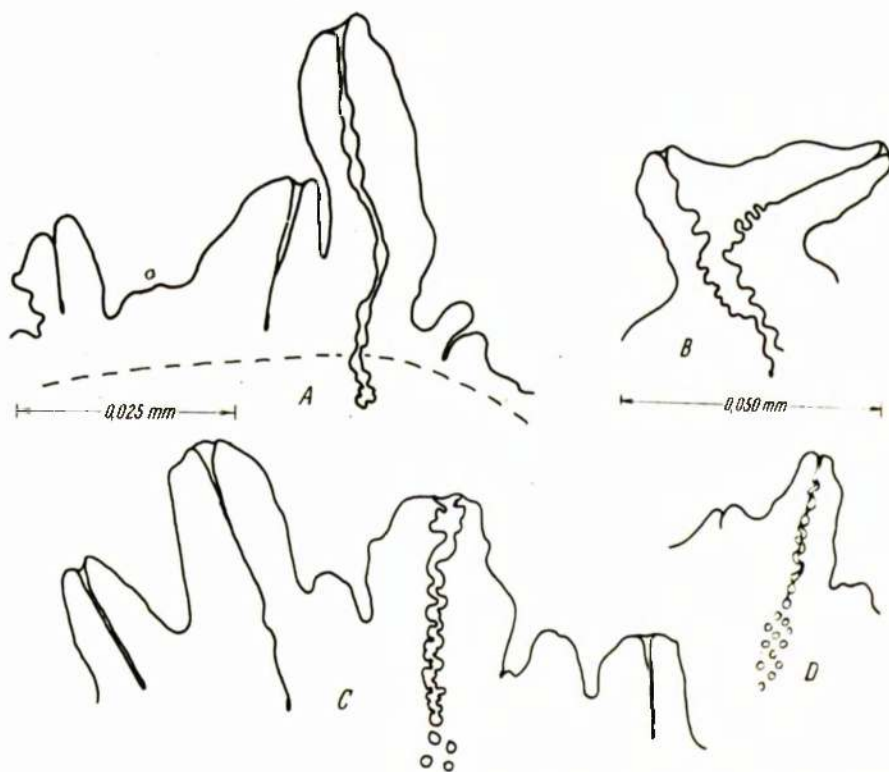
Original reference letters.

- a - alpha granules
- b - beta granules (or mitochondria)
- Ps - plasmasol
- Pg - plasmagel
- H.C - hyaline cap
- N - nucleus
- IV - food vacuole
- C - crystals in vacuoles
- F - spherical masses of substance formed in food vacuoles.

2. Pinocytosis in amoebae induced by egg albumin solution. Channels form from the apices of pseudopods and small droplets fragment from the end of each channel. (Reproduced from Mast and Doyle 1934).



1



2

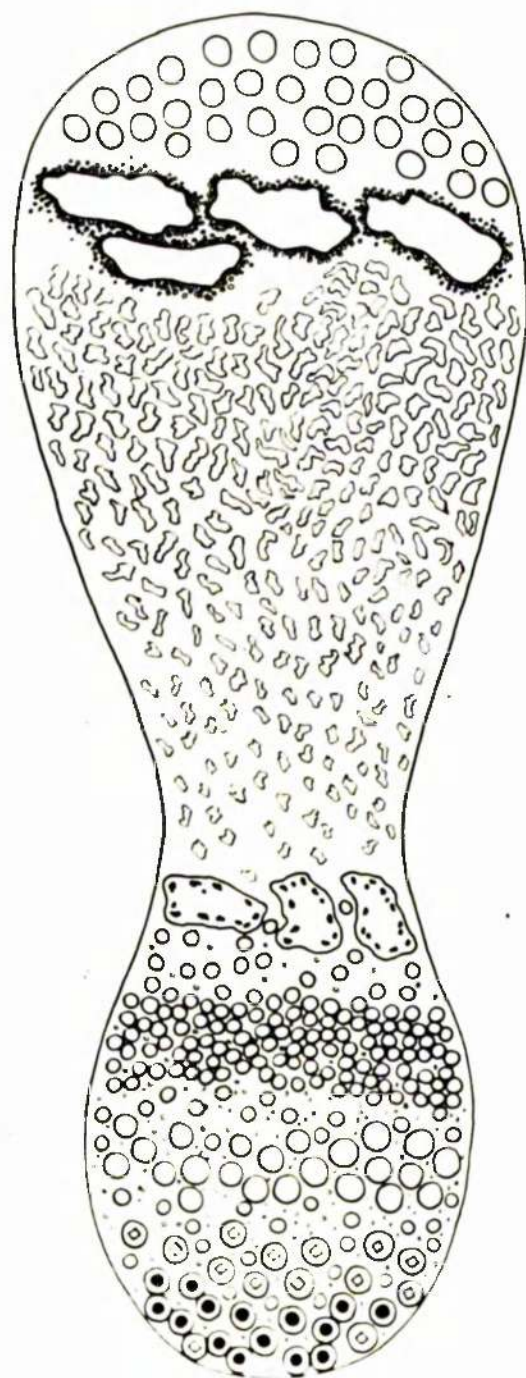
Diagram 3

Impression of centrifuged Pelomyxa
showing the strata the regions are
indicated and are occupied by the
following main structure:-

1. Fat.
2. Contractile vacuoles.
3. Cytomembranes.
4. Nuclei.
5. Mitochondria.
- 6a. Food vacuoles.
- b. Crystals.
- c. Heavy spherical bodies

} plus small round
vesicles or
alpha particles

3



1

2

3

4

5

6a

b

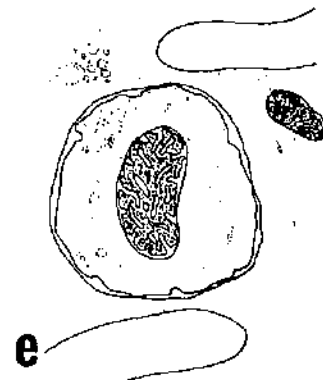
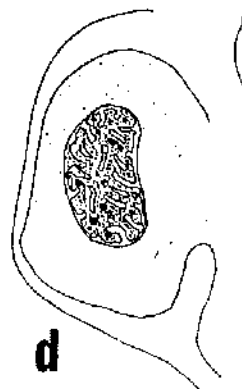
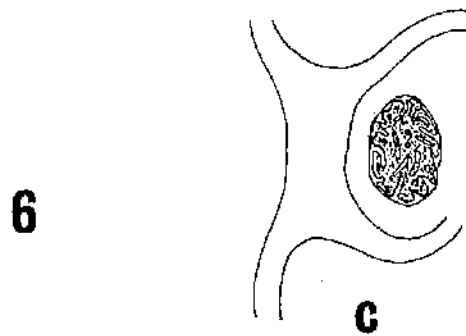
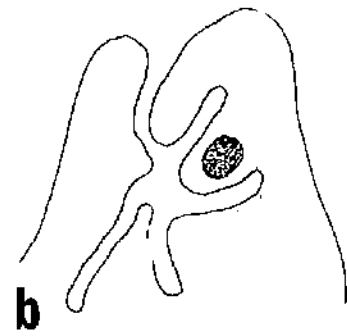
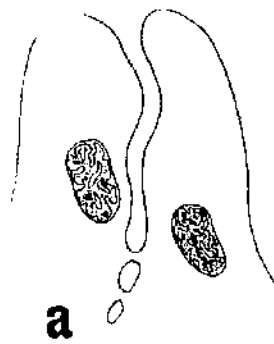
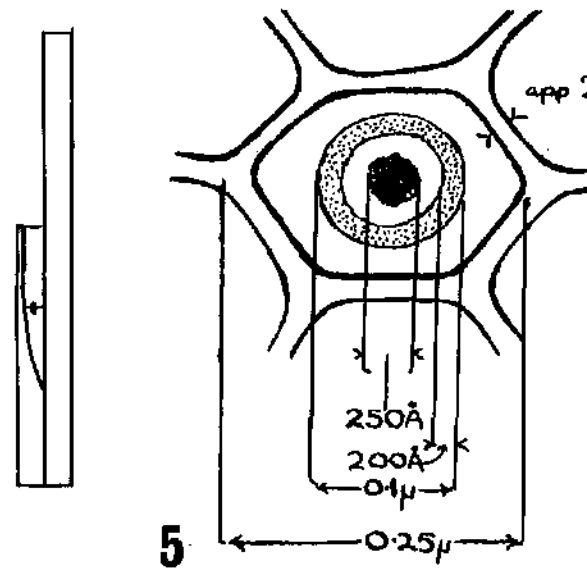
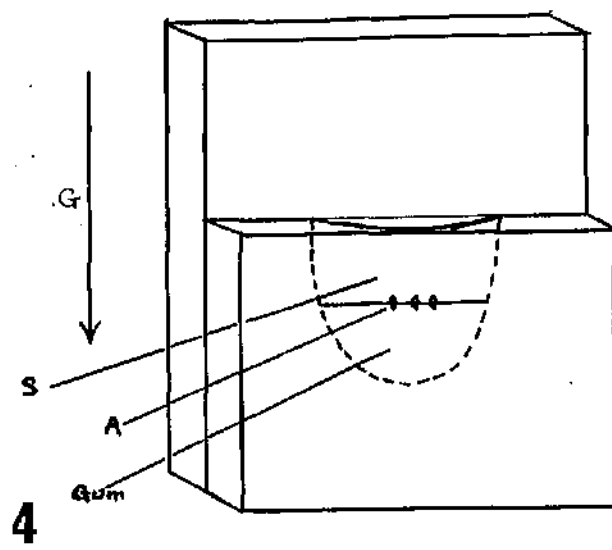
c

Diagrams 4. "Perspex" centrifugation chamber.

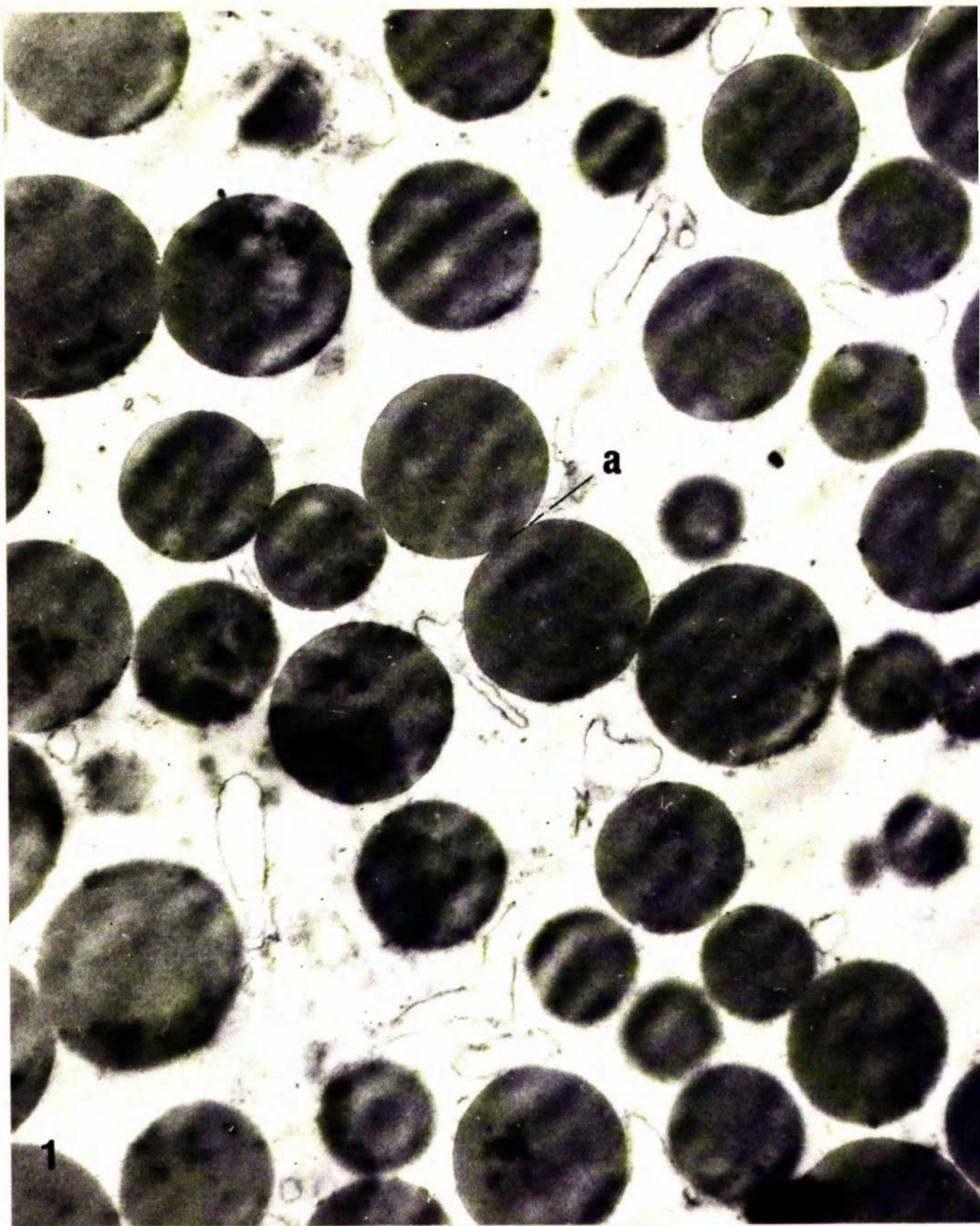
The amoebae (A) are placed at the junction of dialysed gum arabic solution (Gum) and supernatant Pringsheim's solution (S). The gravitational field is indicated by G.

5. The dimensions of the target like structure at the apex of a nuclear honeycomb space (see micrographs 21 - 24).

6. Diagrammatic representation of the process believed to be responsible for sequestration. a,b,c a mitochondrion lies in close contact with a channel. d, during development of the channel, a tongue of cytoplasm containing the mitochondrion is surrounded by the channel and its membranes. e, when the channel fragments, the mitochondrion and its cytoplasm are isolated between two concentric membranes (see micrographs 95,96 and 97 - 102).

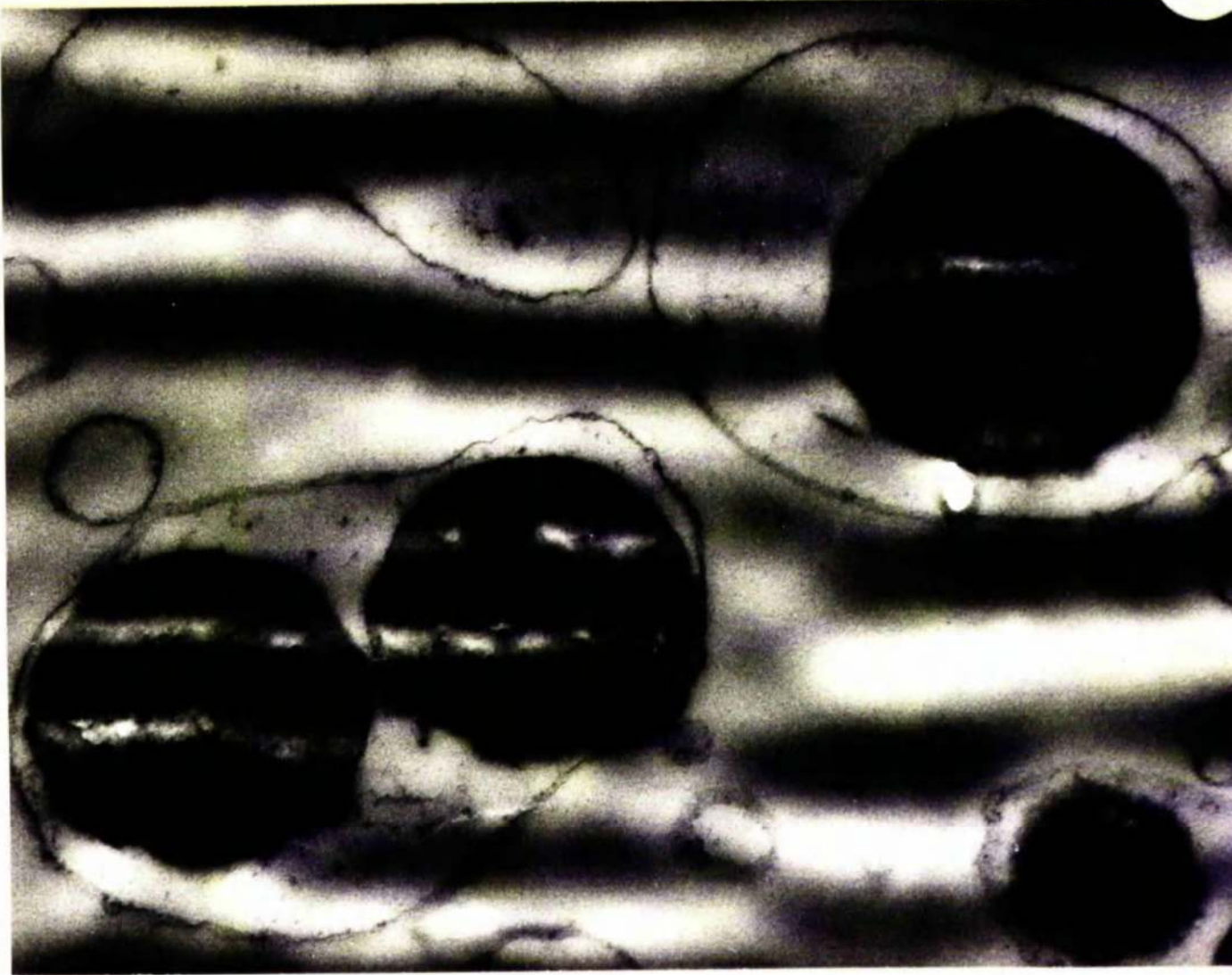


Micrograph 1 From region 1 of centrifuged
Pelomyxa. There are large osmiophilic fat
droplets and occasional small membranous
vesicles in between. The fat droplets are
in contact in places (a) but retain their
spherical shape. (mag. 17,000).



Micrograph 2 Part of region 1 showing small fat droplets enclosed by membranes. This is a very poor section showing gross cutting artefact. The appearance of membrane enclosed fat droplets is very rare and has been seen definitely only in centrifuged amoebae. (mag. 26,000).

Micrograph 3 Periphery of region 3 - 4 after staining with phosphotungstic acid solution. The plasma membrane is heavily intensified with easily visible outer filaments. The cytoplasm has a very granular appearance and small irregular vesicles intensify with the stain. They represent micropinocytotic vesicles of uncertain origin. (mag. 22,000).



2



3

Micrograph 4 Region 2. A contractile vacuole with attendant satellite vesicles (Ve) which although close to the wall are not in continuity with it. A few cytomembranes (cm) lie near the vacuole but there are no mitochondria (see micrograph 29). The lumen contains some tiny granules of unknown origin. (mag. 27,000).



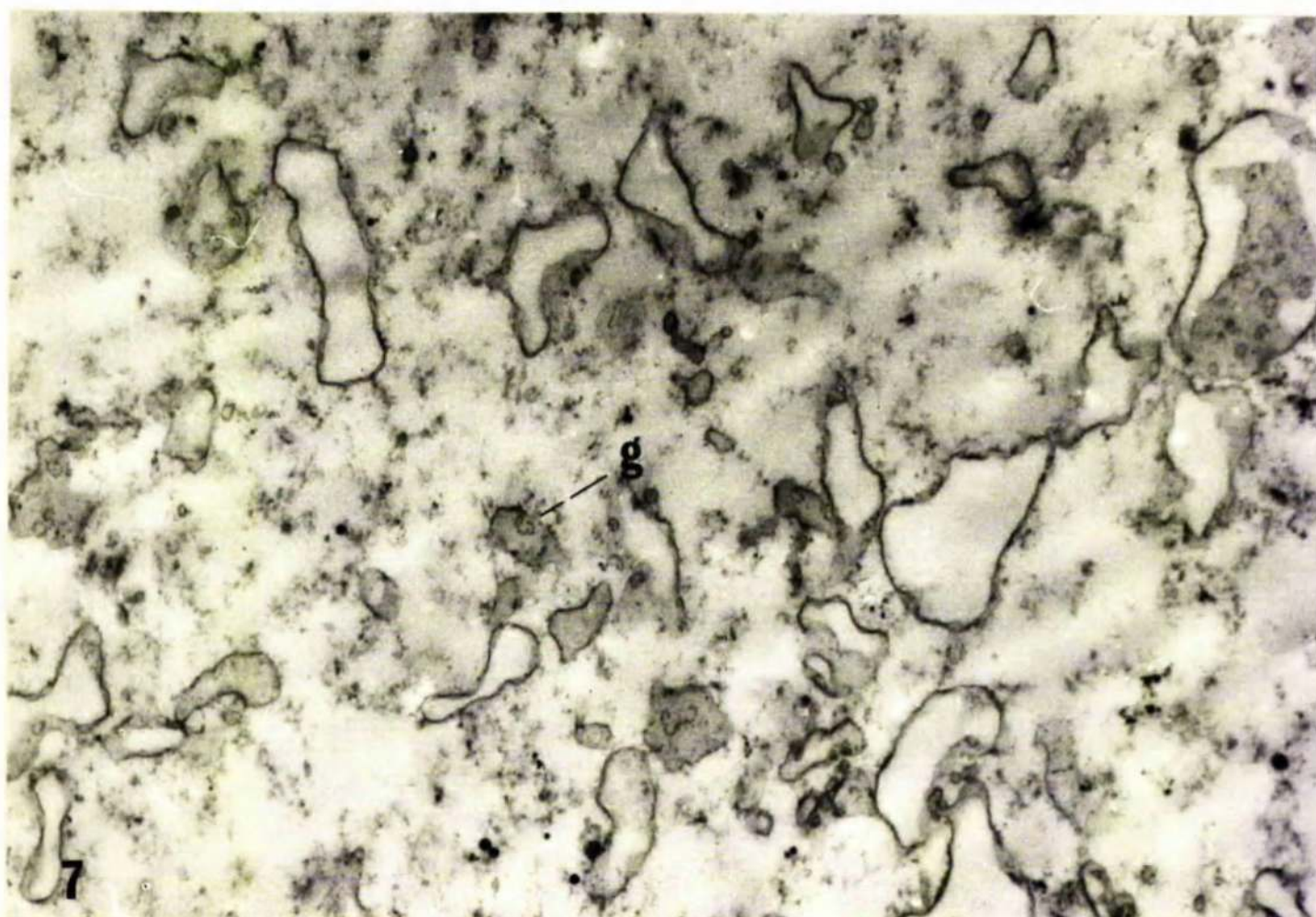
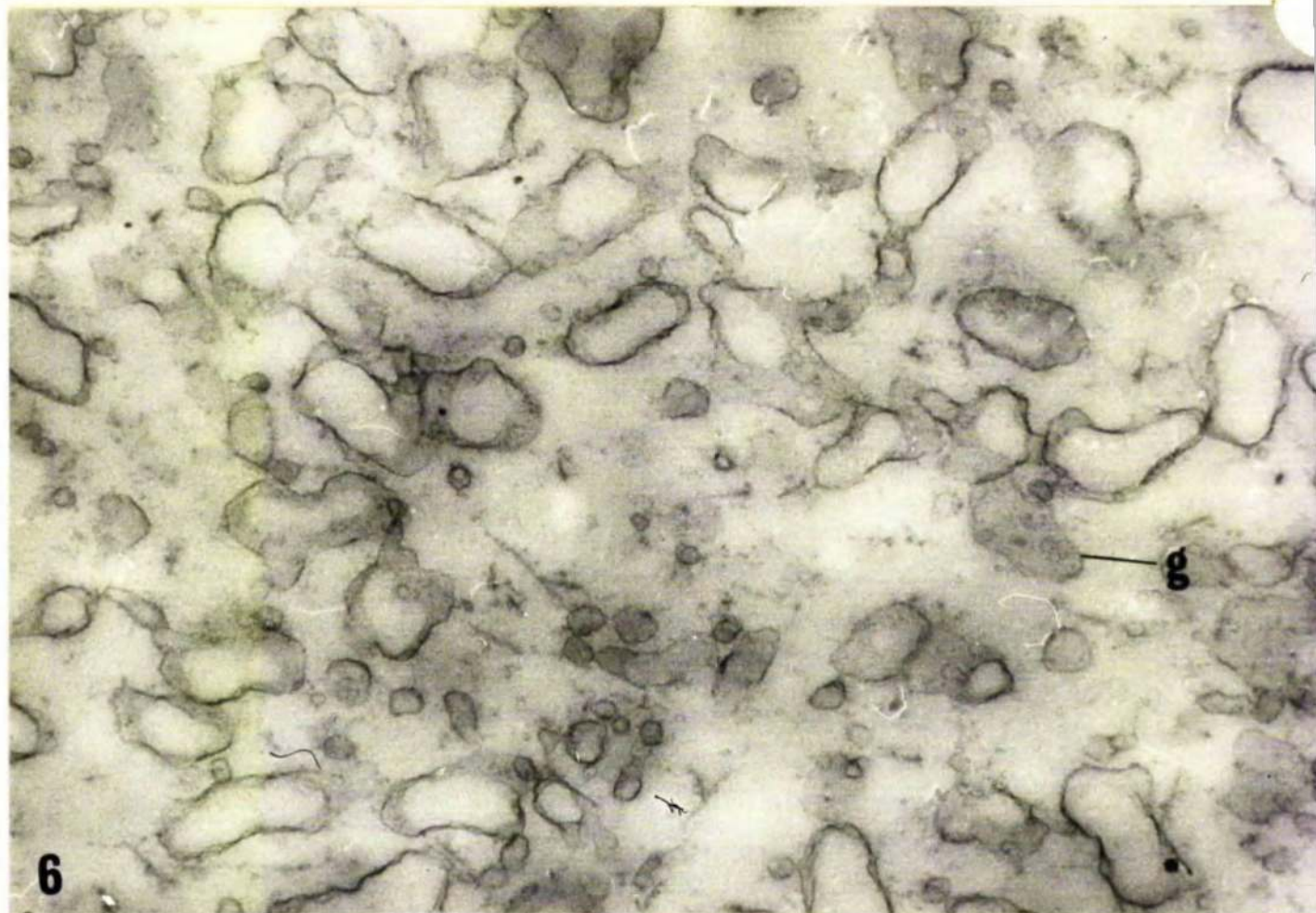
Micrograph 5 Channels probably of pinocytotic origin are found near the contractile vacuoles between regions 3 and 2. They are lined with plasma membrane with a filamentous layer. The surrounding cytoplasm is granular and should be compared with micrograph 72 . Cytomembranes surround the granular area. (mag. 7,500).



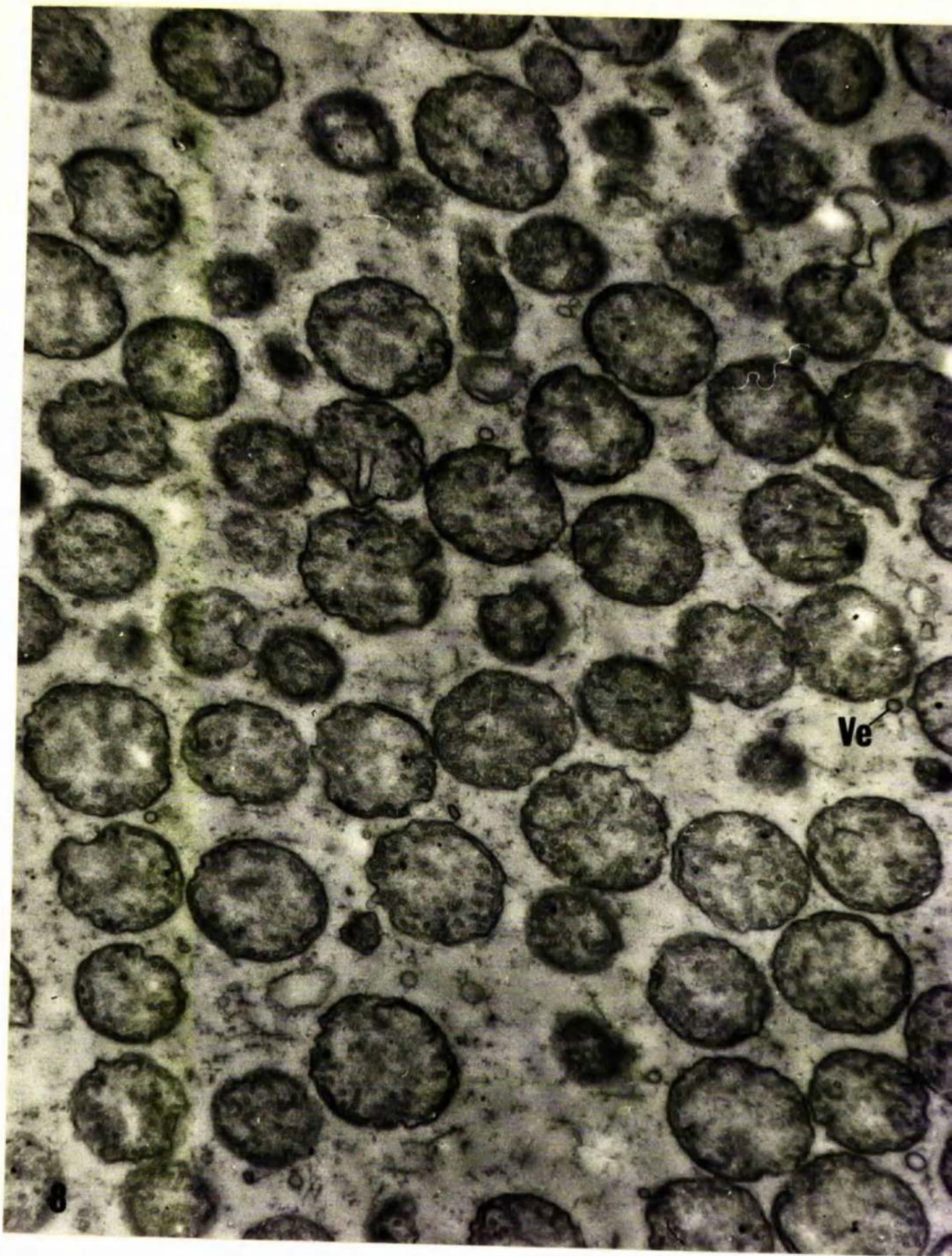
Micrograph 6 Region 3. The cytomembranes.

The walls are rarely cut at right angles because of the extreme irregularity of shape. Only a few pale granules are visible in tangential section (g). (mag. 24,000).

Micrograph 7 Cytomembranes after lead staining by Karnowsky's method. The overall contrast is increased and the granules are easily visible forming whorls in tangential section (g). (mag. 20,000).

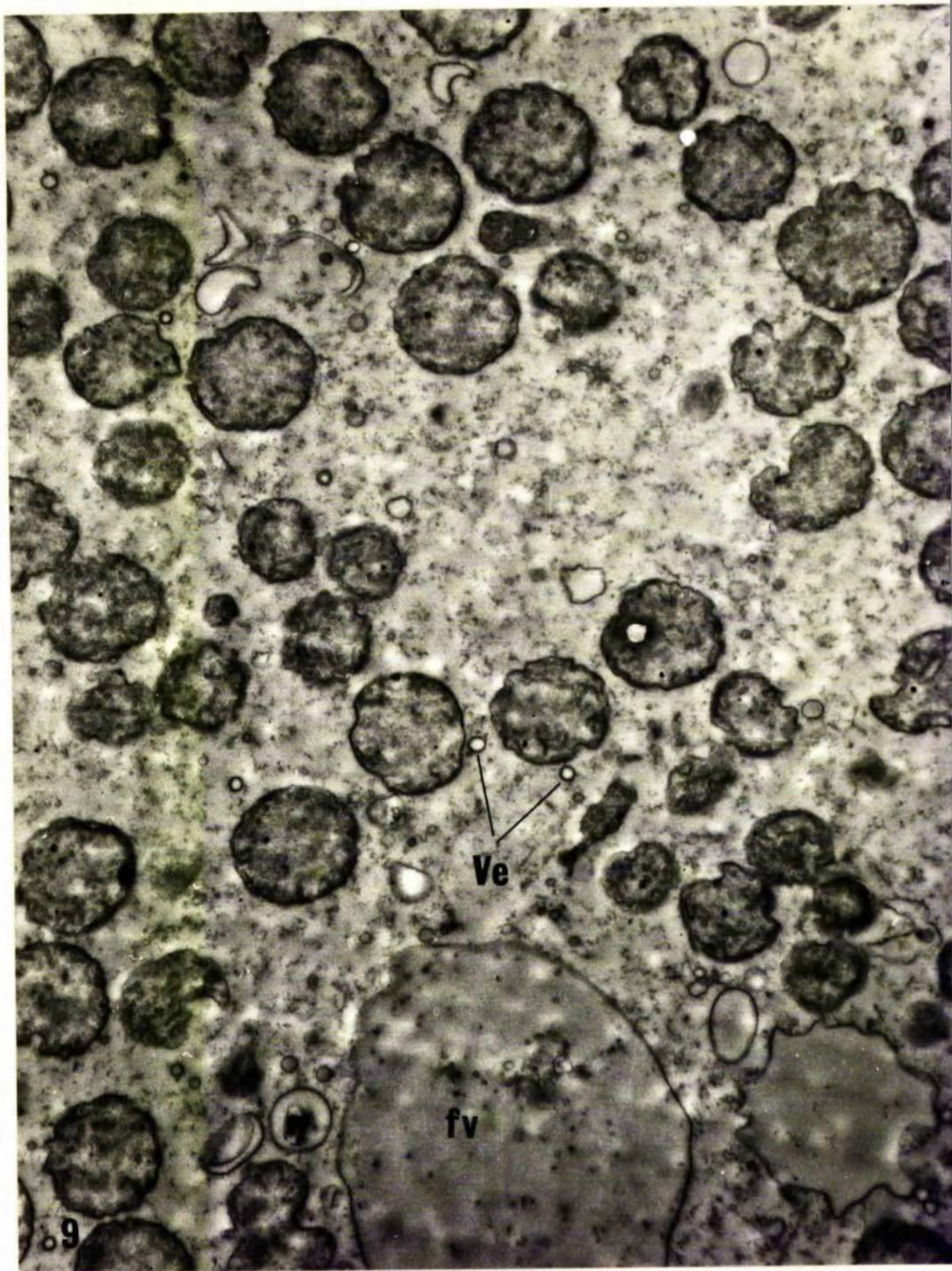


Micrograph 8 The main mitochondrial band
(region 5). Typical, close packed, tubular
mitochondria with clear intervening cytoplasm.
There are a few small round vesicles in the
region (Ve). (mag. 15,000).

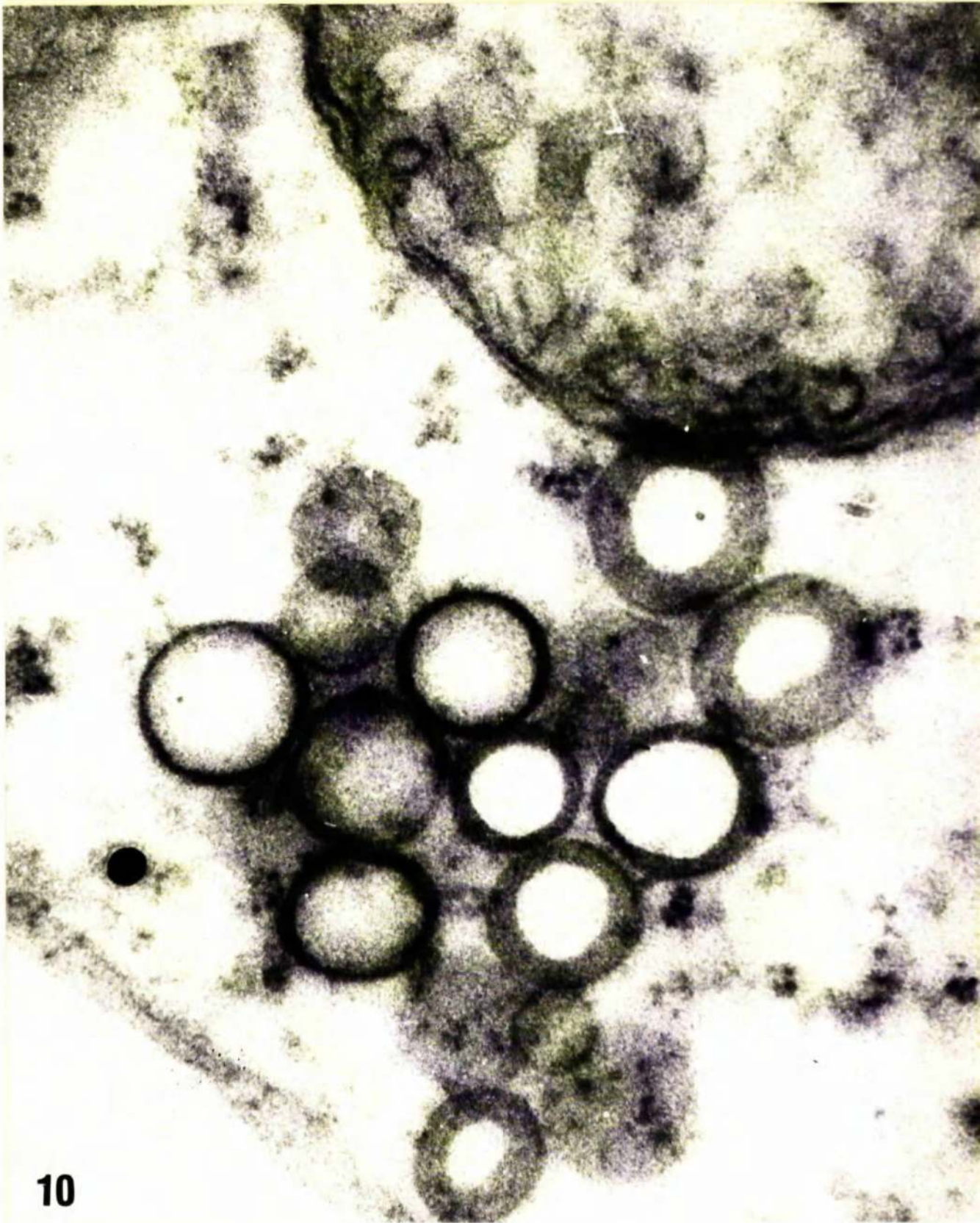


Micrograph 9 More centrifugally in the region 5.

The mitochondria are more sparse and there is more granular background cytoplasm. The lightest food vacuoles (fv) are found in this region and small round vesicles are more common (Ve). (mag. 13,000).

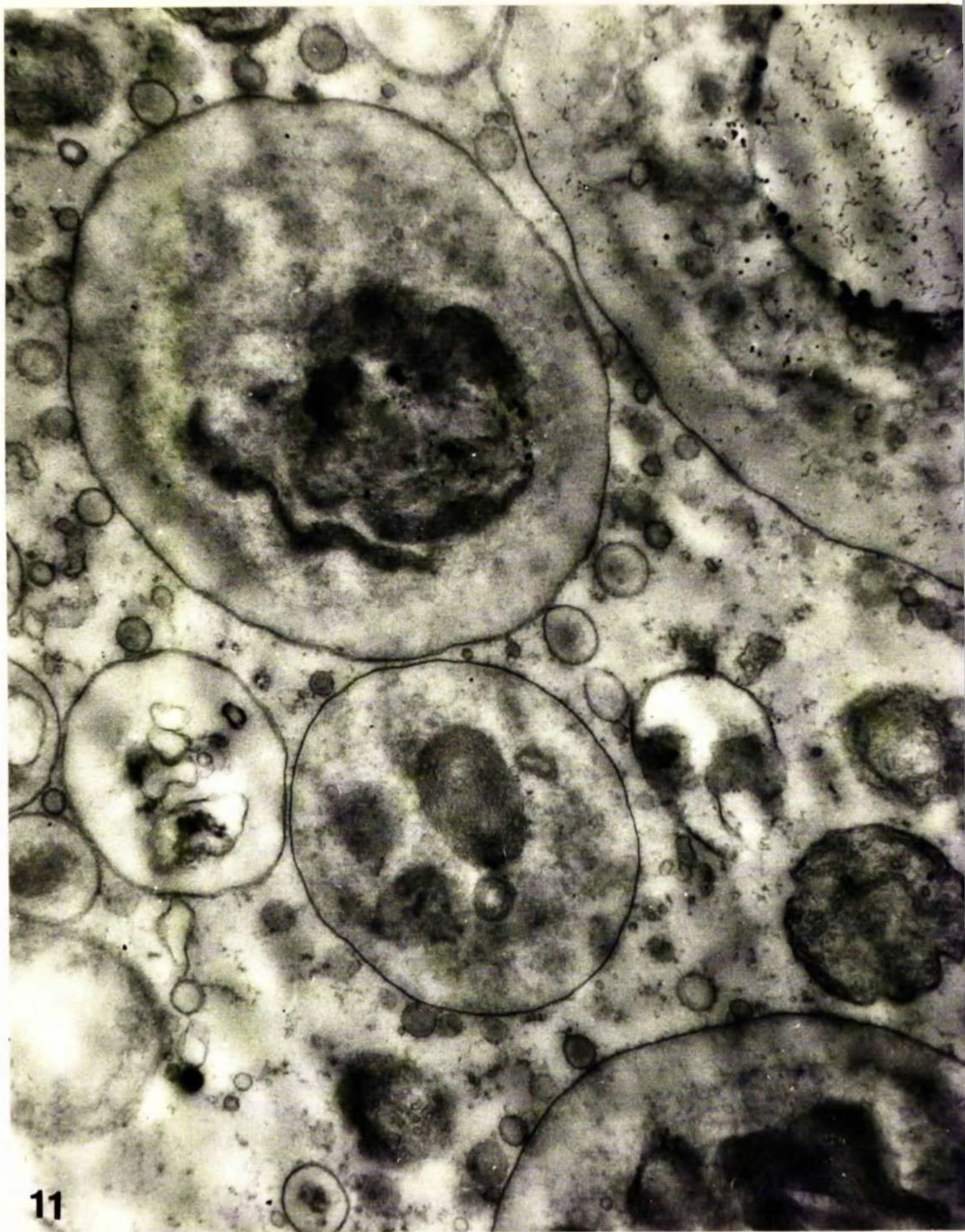


Micrograph 10 A higher magnification of the small round vesicles of the centrifugal mitochondrial zone. Their size is uniform but the apparent thickness of the wall varies as a result of the differing incidence of the section. The thickness of the section in this case is about 800 Å and the vesicles are about 2,000 Å in diameter. The overlapping of two vesicles cut tangentially is also due to the section thickness. (mag. about 77,000).

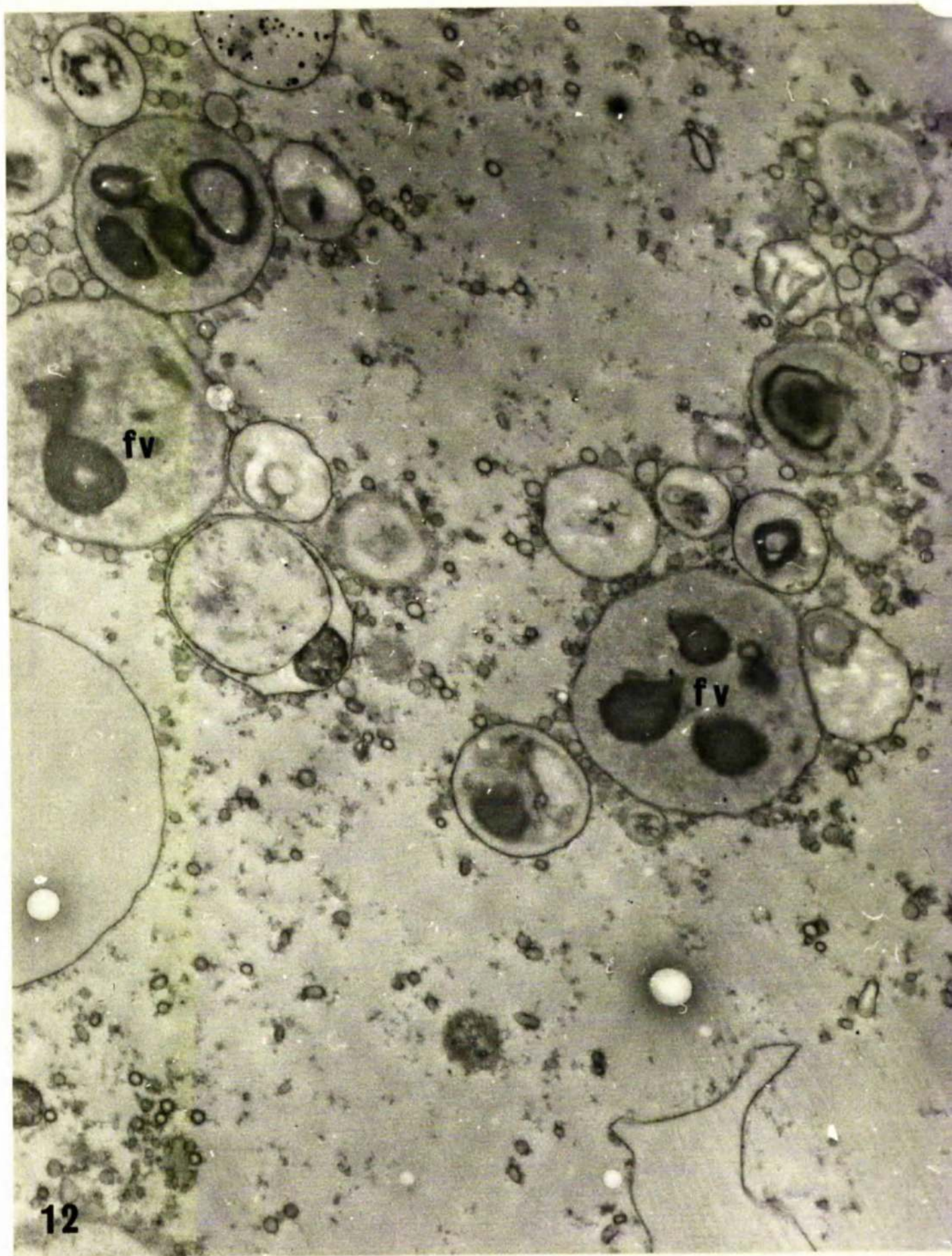


10

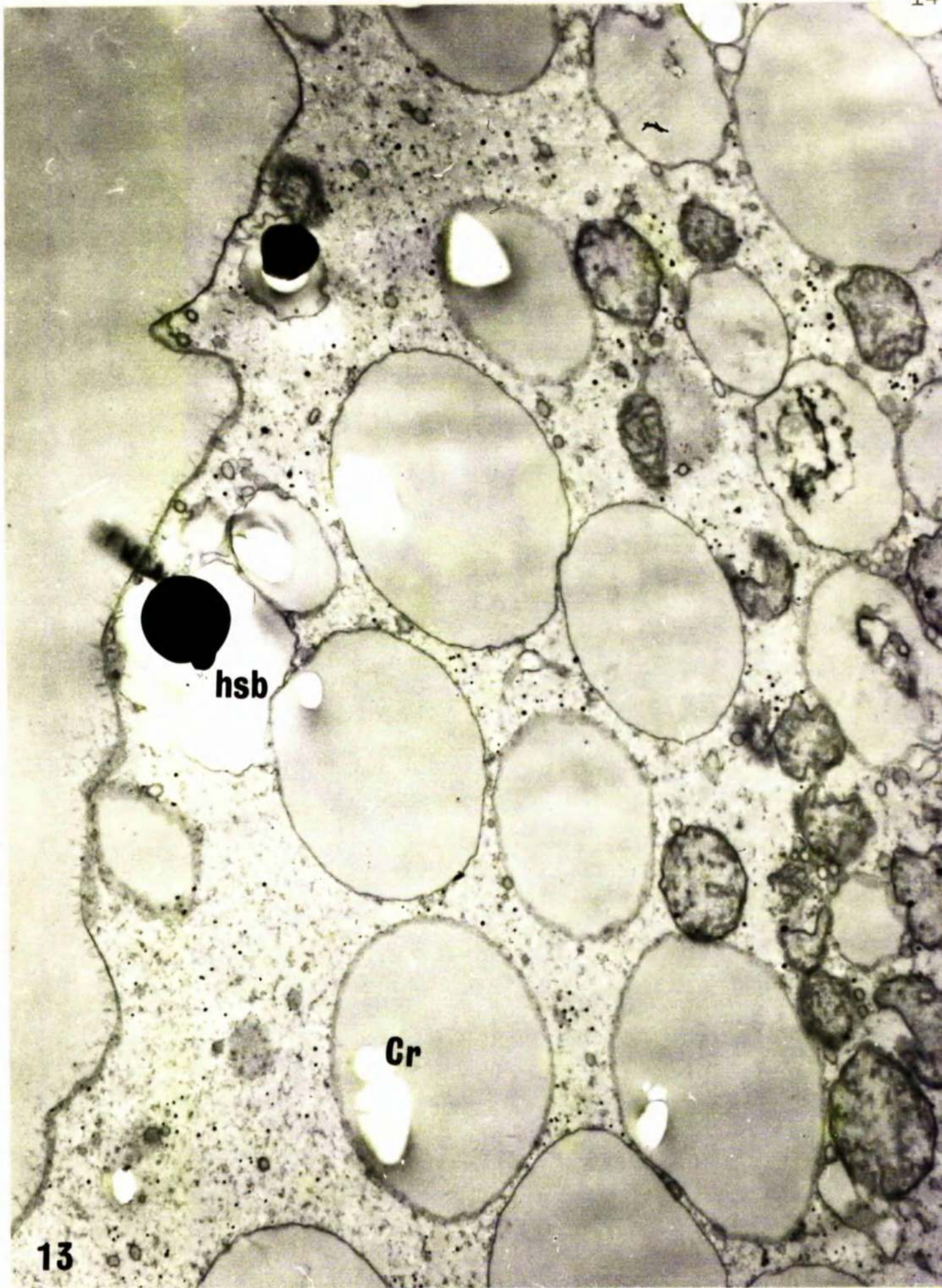
Micrograph 11 Region 6a - the food vacuole zone. Each vacuole contains granular ground substance with a more or less empty periphery. The central mass consists largely of a lamellated substance. The cytoplasm contains round satellite vesicles of a wide range in diameter. (mag. 23,000).



Micrograph 12 Region 6a contains large food vacuoles (fv) with fragments of food as well as central lamellated masses believed to be of phospholipid. There are large numbers of small round vesicles or alpha particles both as satellites to food vacuoles and free in the intervening cytoplasm. (mag. 10,000).



Micrograph 13 Region 6b and c. Crystals show as translucent negatives (Cr) within smooth round vacuoles. Some vacuoles with a similar structure contain no crystals, either because they lie outside the section or because none is present. The heaviest pole, in this particular section very thin, contains heavy spherical bodies (hsb) with their intensely electron dense core and eccentric translucent halo. The plasma membrane has a visible filamentous layer. (mag. 9,500).



Micrograph 14 Heavy spherical bodies showing the damage resulting from either sectioning or the electron beam. There are also one or two small spherical vesicles present. The plasma membrane has a distinct filamentous layer. (mag. 22,000).

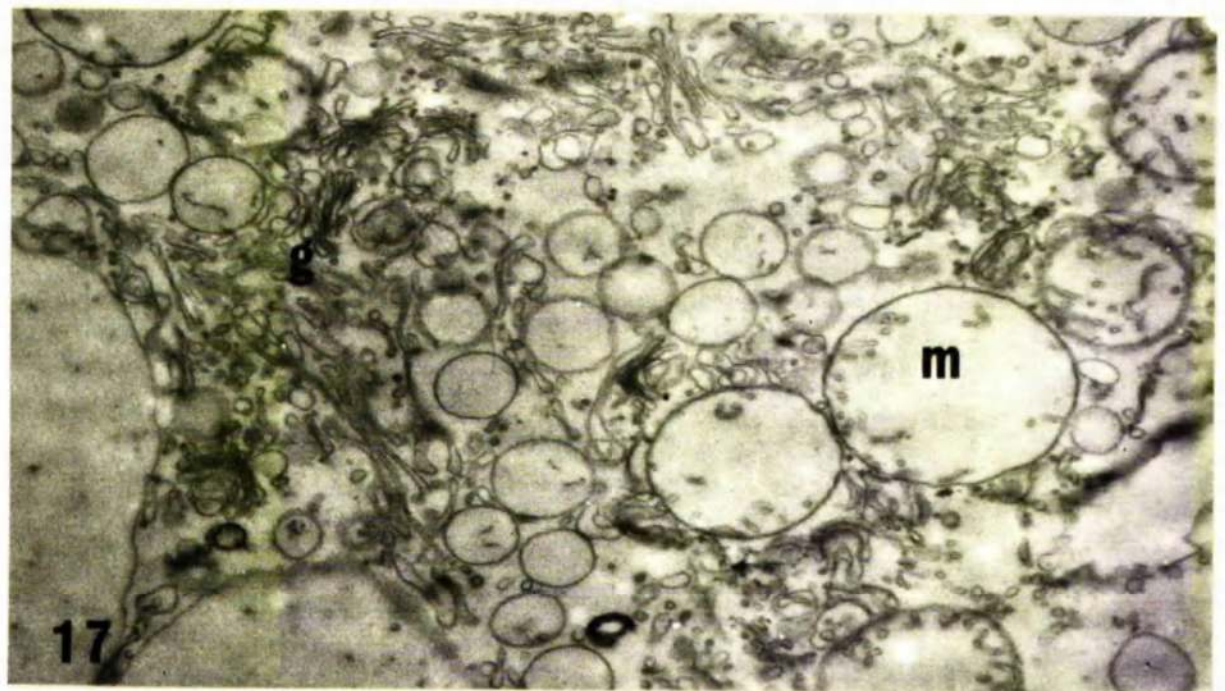
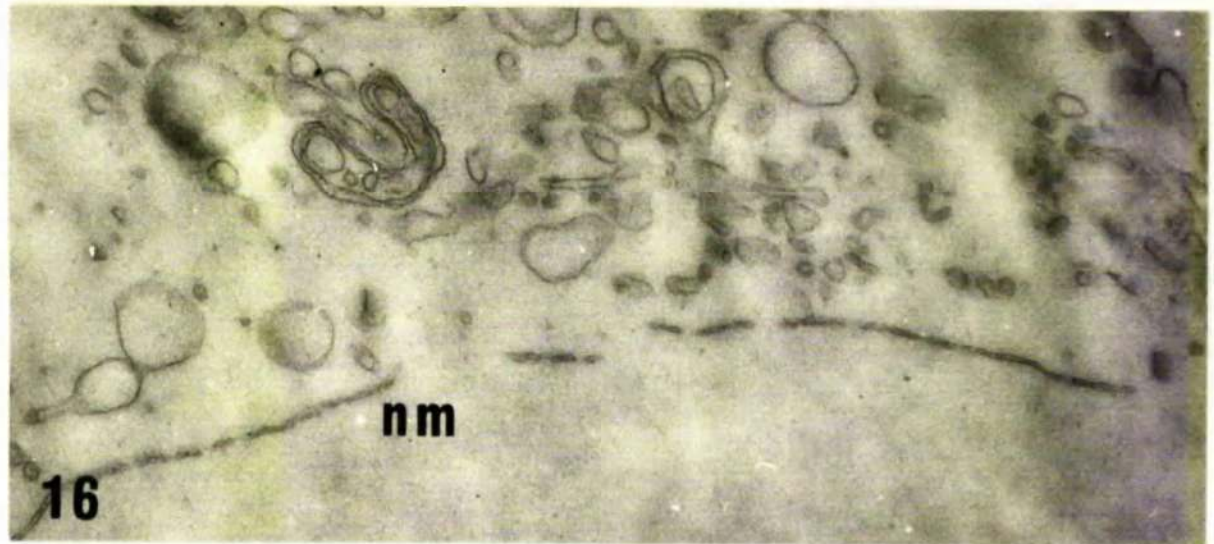
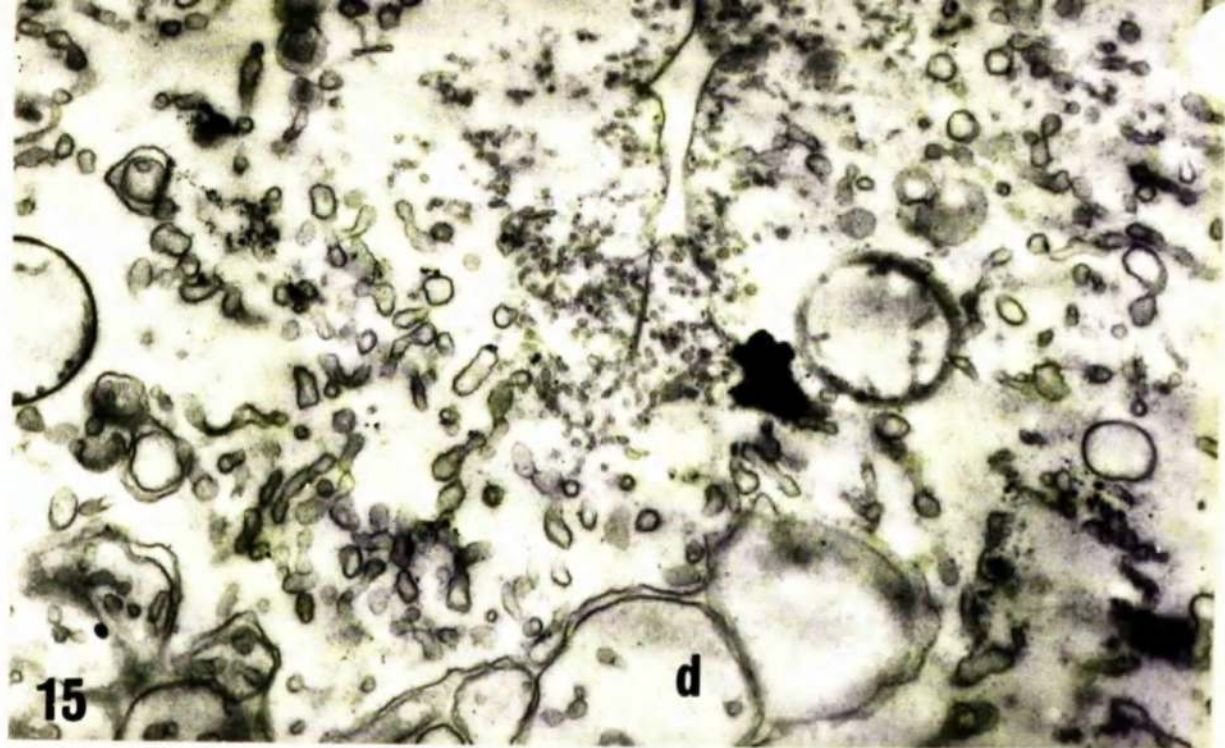


14

Micrograph 15 Region 2 and 3 after potassium permanganate fixation. The effect is generally one of decreased irregularity of membrane as if by swelling or rounding off, however, the dimensions of individual organelles are if anything reduced. There is overall loss of granulation both in cytoplasm and on cytomembranes. The structure d is commonly found - it consists of enclosed membranes and cannot be related to any recognised structure. (mag. 9,000).

Micrograph 16 Region 4 after permanganate. The nuclear membrane (nm) is discontinuous (compare with micrograph 19). Enclosed membranous structures are seen again. (mag. 18,000).

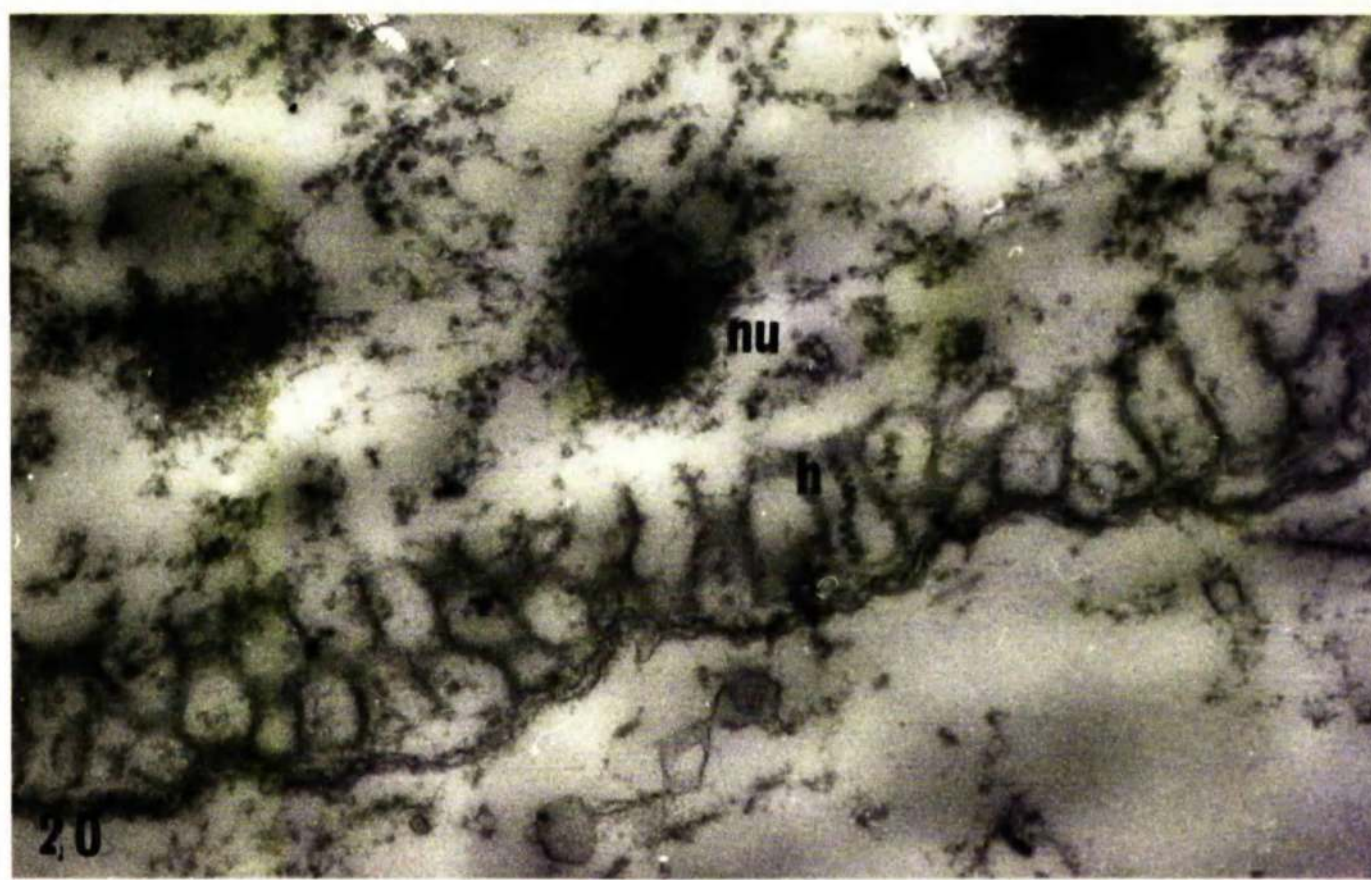
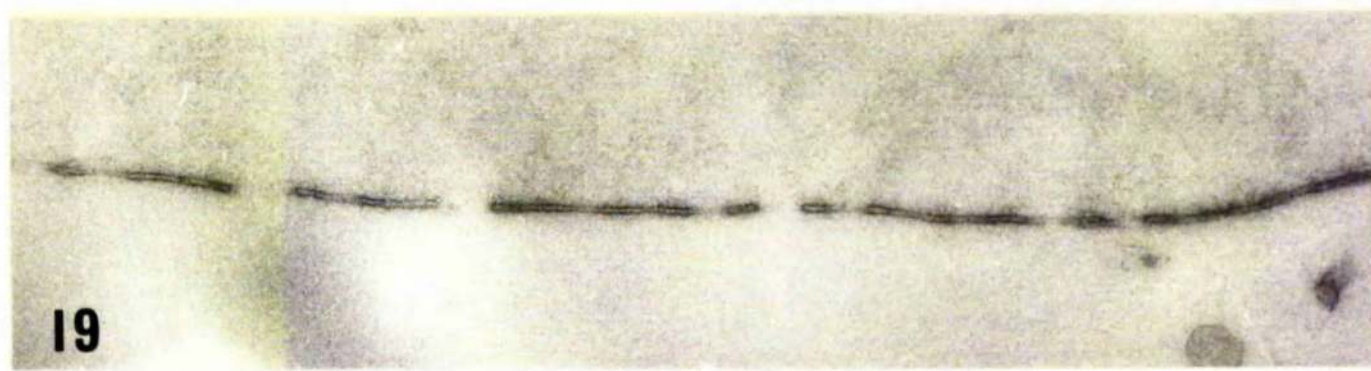
Micrograph 17 Golgi region after permanganate. Golgi membranes (g) have not been seen in osmium fixed specimens of centrifuged material. The mitochondria (m) are grossly altered with few tubules. The remaining vesicular structures may be swollen alpha particles but have not been certainly identified. (mag. 26,000).



Micrograph 18 Plasma membrane of Amoeba proteus
after permanganate fixation. The filamentous
layer has disappeared and the plasmalemma proper
consists of a triple layered or unit membrane
structure. (mag. 77,000).

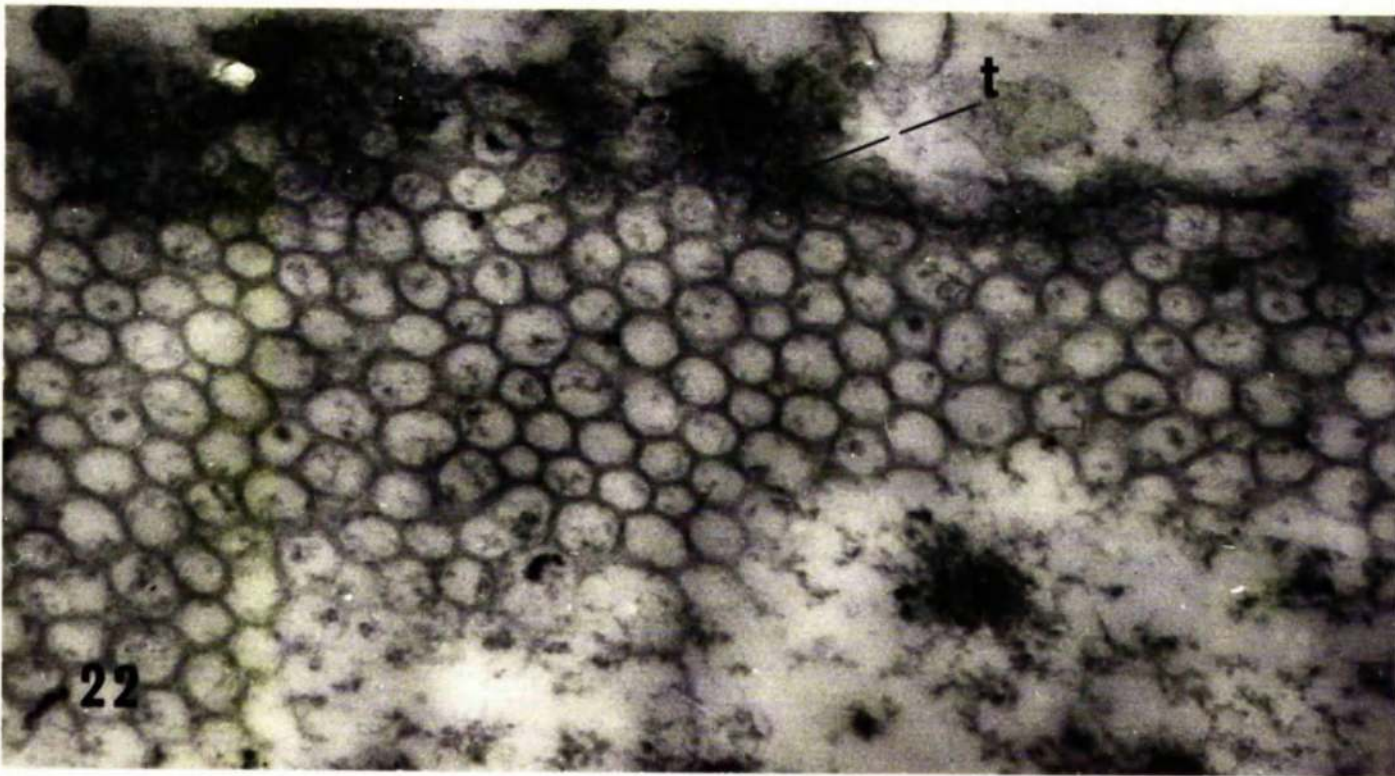
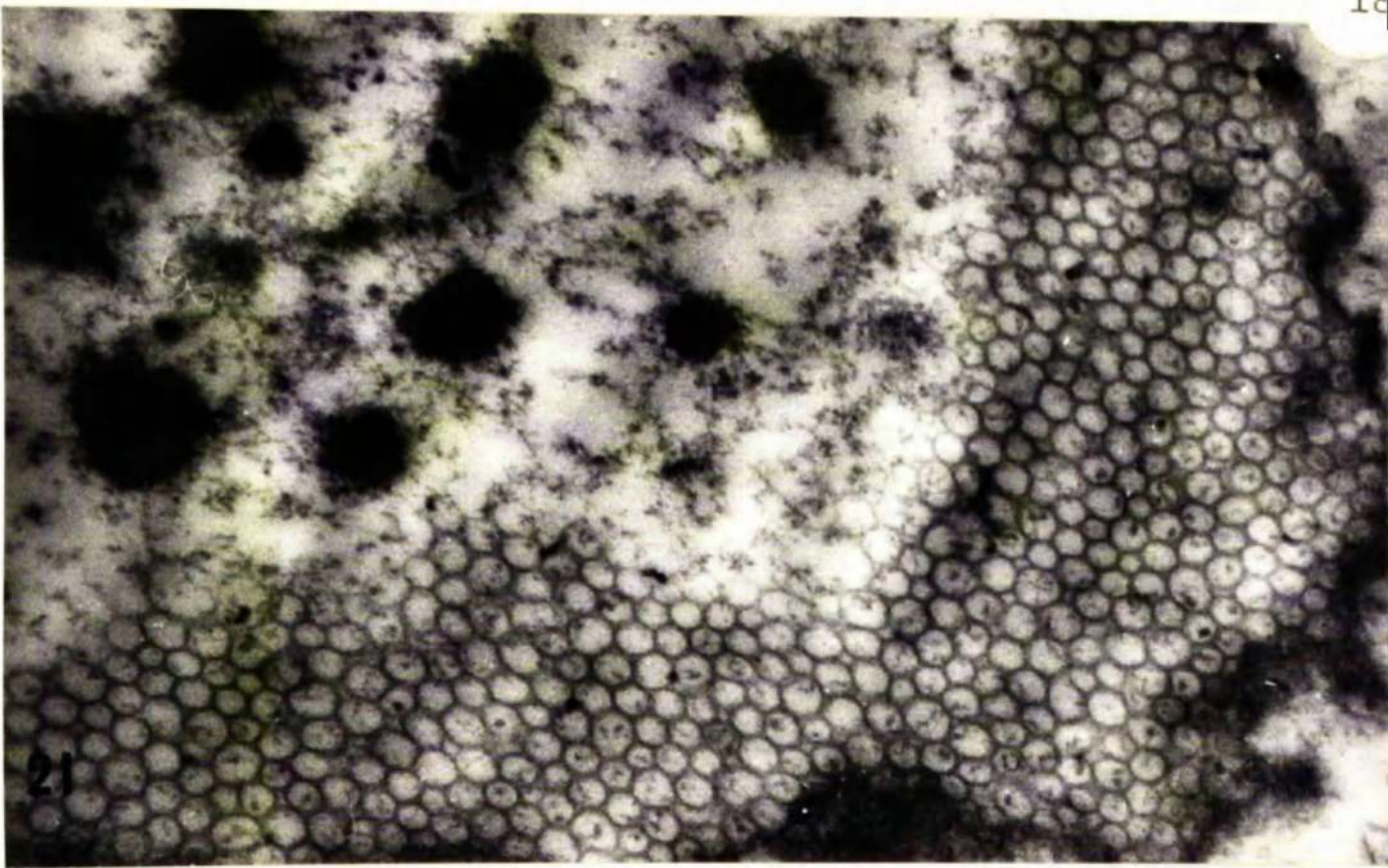
Micrograph 19 Nuclear membrane of Amoeba proteus.
The two outer membranes are visible but are
discontinuous; the "honeycomb" is not seen and
the nucleoplasm immediately beneath the membrane
is only faintly granular. (mag. appx. 30,000).

Micrograph 20 Nuclear membrane of Amoeba proteus
after osmium fixation. There is an irregular
double outer membrane from which vertical partitions
of double membranes project into the nucleoplasm to
form the "honeycomb". A nuclear helix (of Pappas)
can be seen inside one of the spaces (h).
nu = nucleolus. (mag. 32,000).

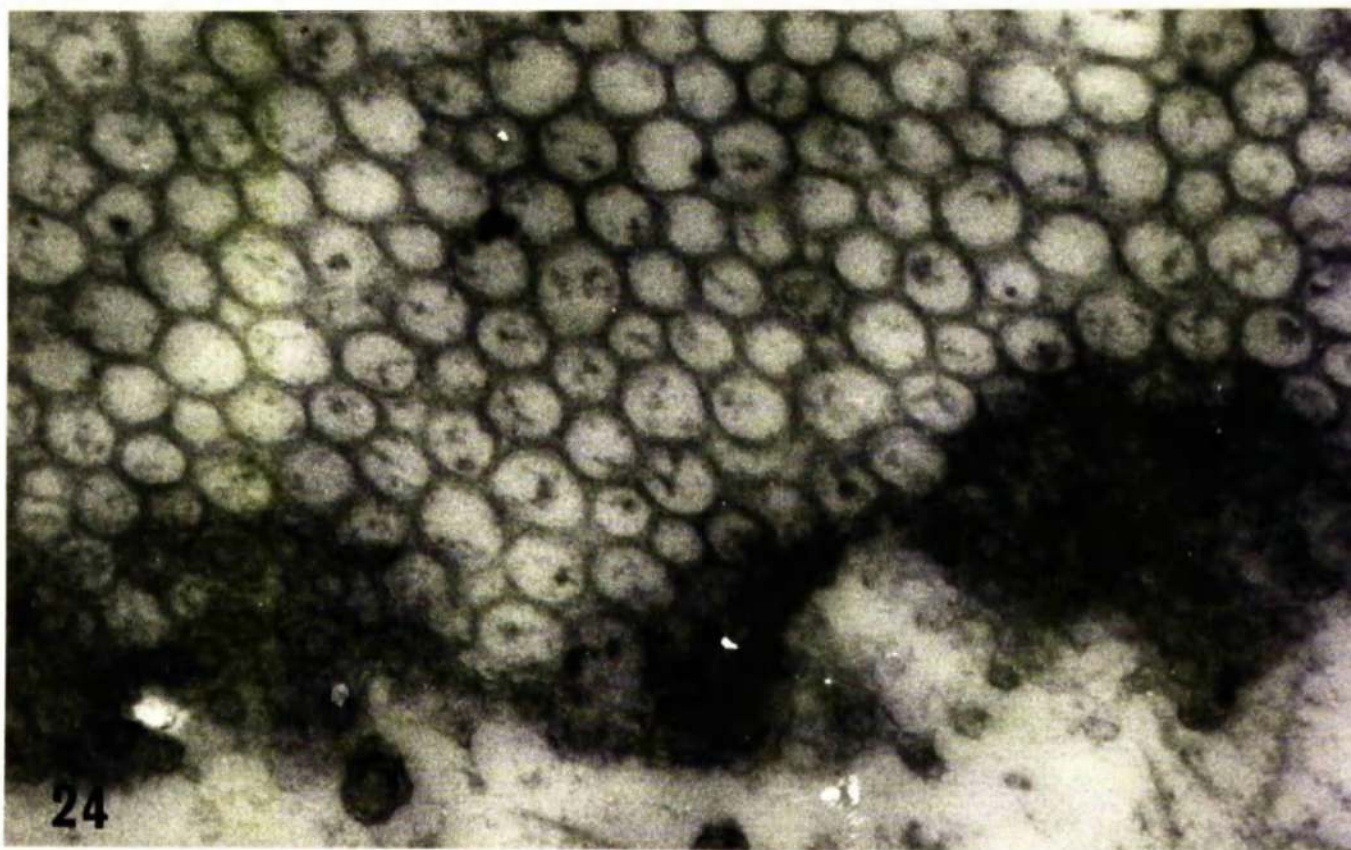
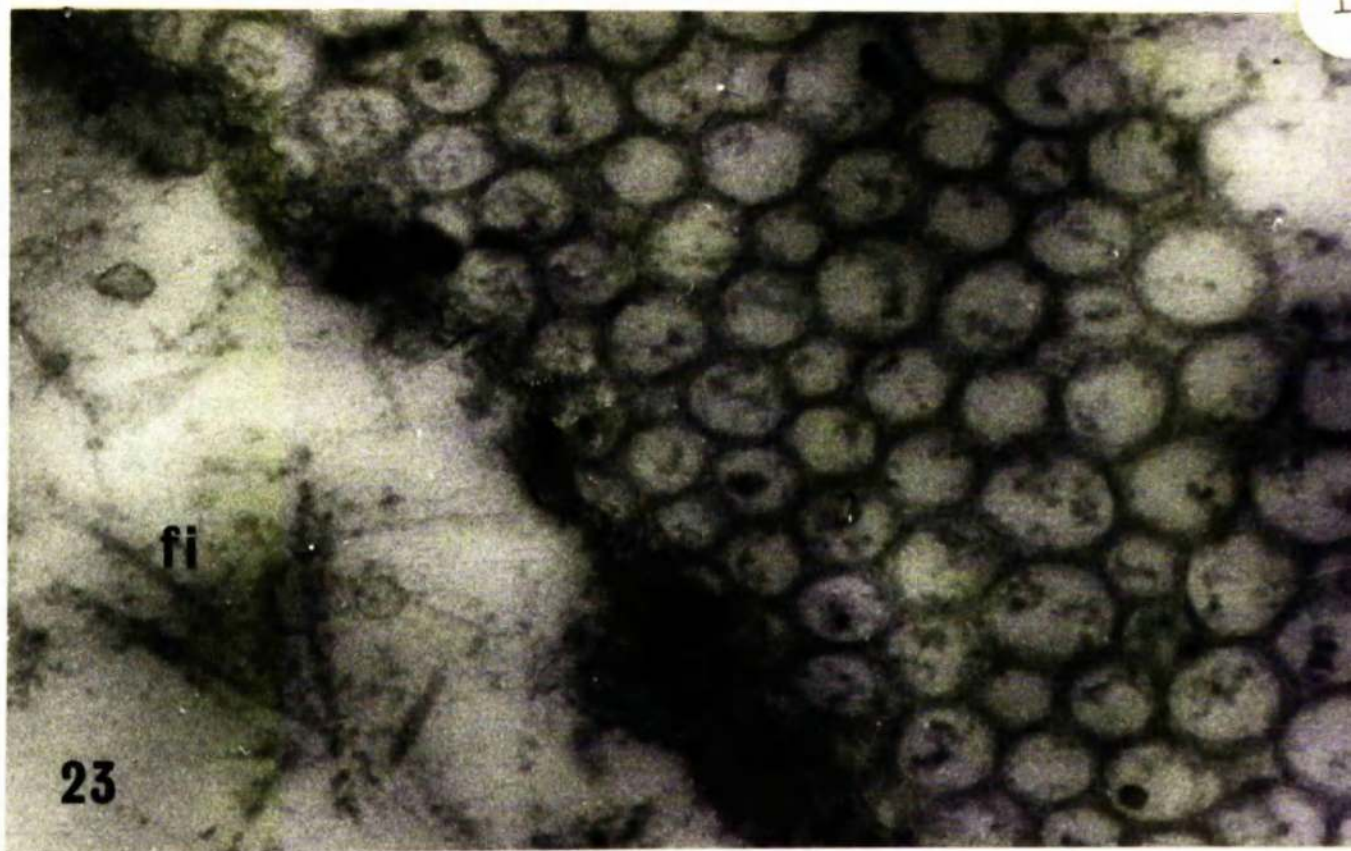


Micrograph 21 Almost tangential section through nuclear "honeycomb" showing the general layout of the spaces. The peripheral nucleolar masses can be seen. (mag. 16,000).

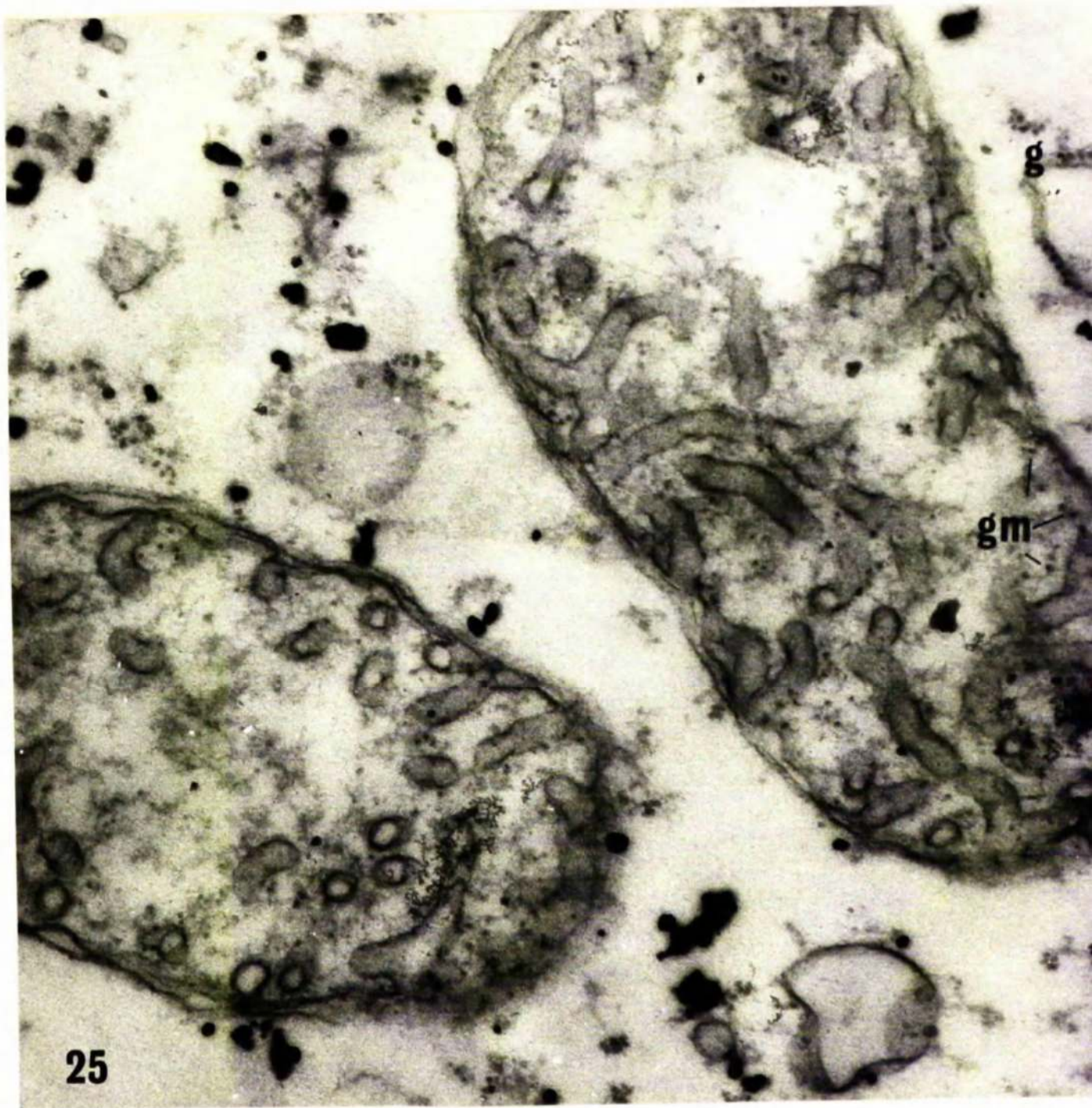
Micrograph 22 Similar to 21. The target-like configuration can be seen at the apex of a space (t). Its dimensions are given in diagram 5 (mag. 28,000).



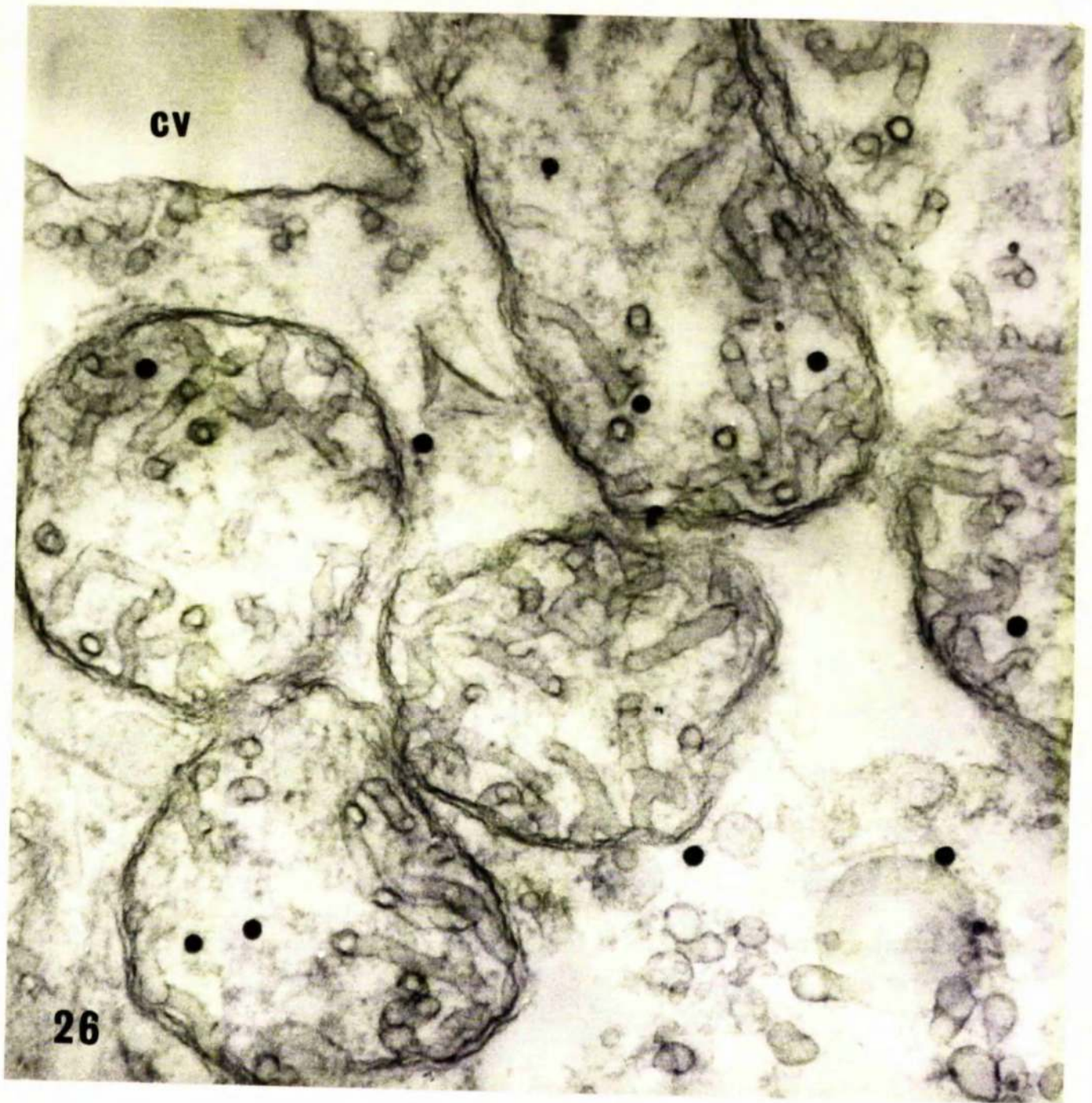
Micrographs 23 and 24 Further tangential sections through nuclear membranes with the same details. In 23 there are short straight fibrils around the nucleus (f1) of the type seen during mitosis. In 24 there is a double walled vesicle of the type which might have been produced by blebbing from the nuclear membrane. (mag. 58,000 and 35,000).



Micrograph 25 Normal mitochondria of Amoeba
proteus in a section stained with Karnovsky's
lead solution. The cytomembranes have prominent
granules (g) and there are similar granules
within the mitochondrial matrix (gm). There is
some lead contamination which is quite distinct
because of its random deposition. The cytoplasm
also contains dense debris of unidentified origin
which occurs equally well without staining. The
mitochondrial structure is as discussed in the
text. (mag. 50,000).

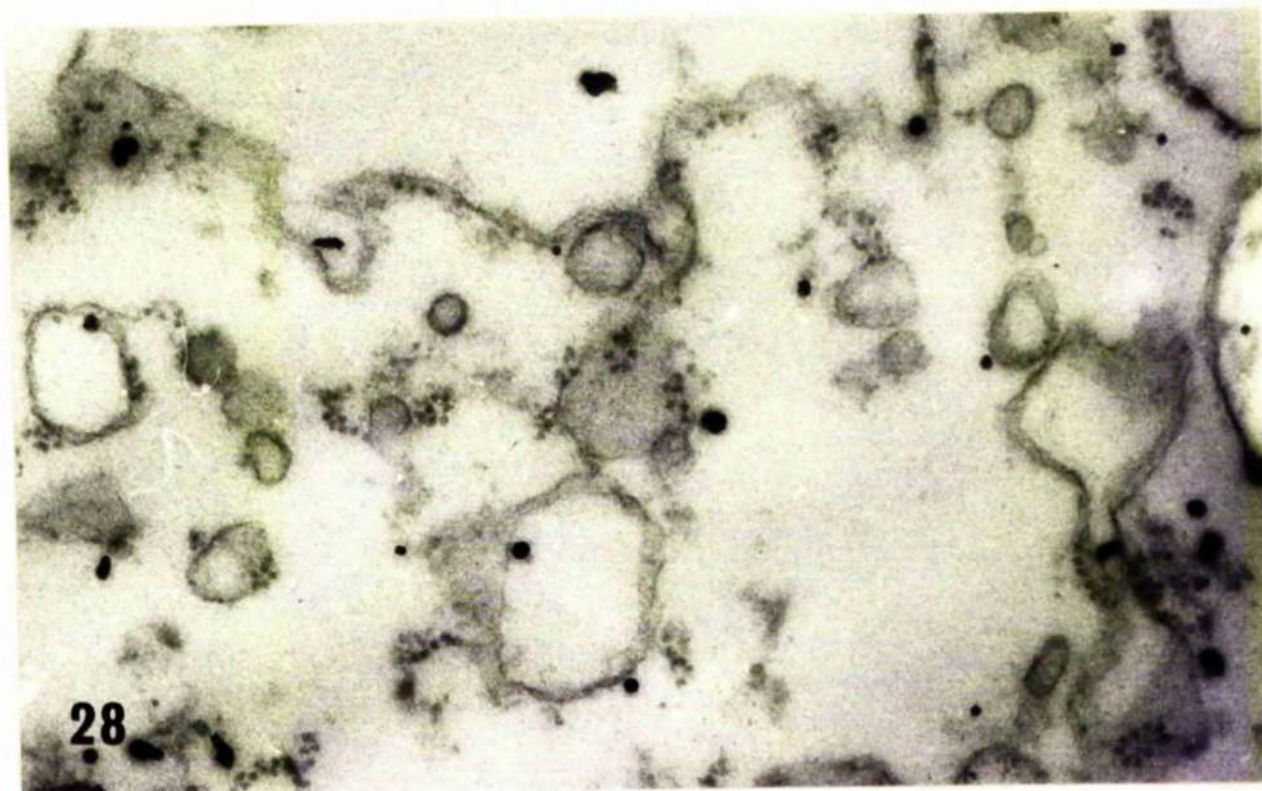


Micrograph 26 Mitochondria surrounding a contractile vacuole (cv) unstained. The structure is identical with micrograph 25. The tubules are continuous with the outermost double membranes in a number of places. (mag. 33,000).

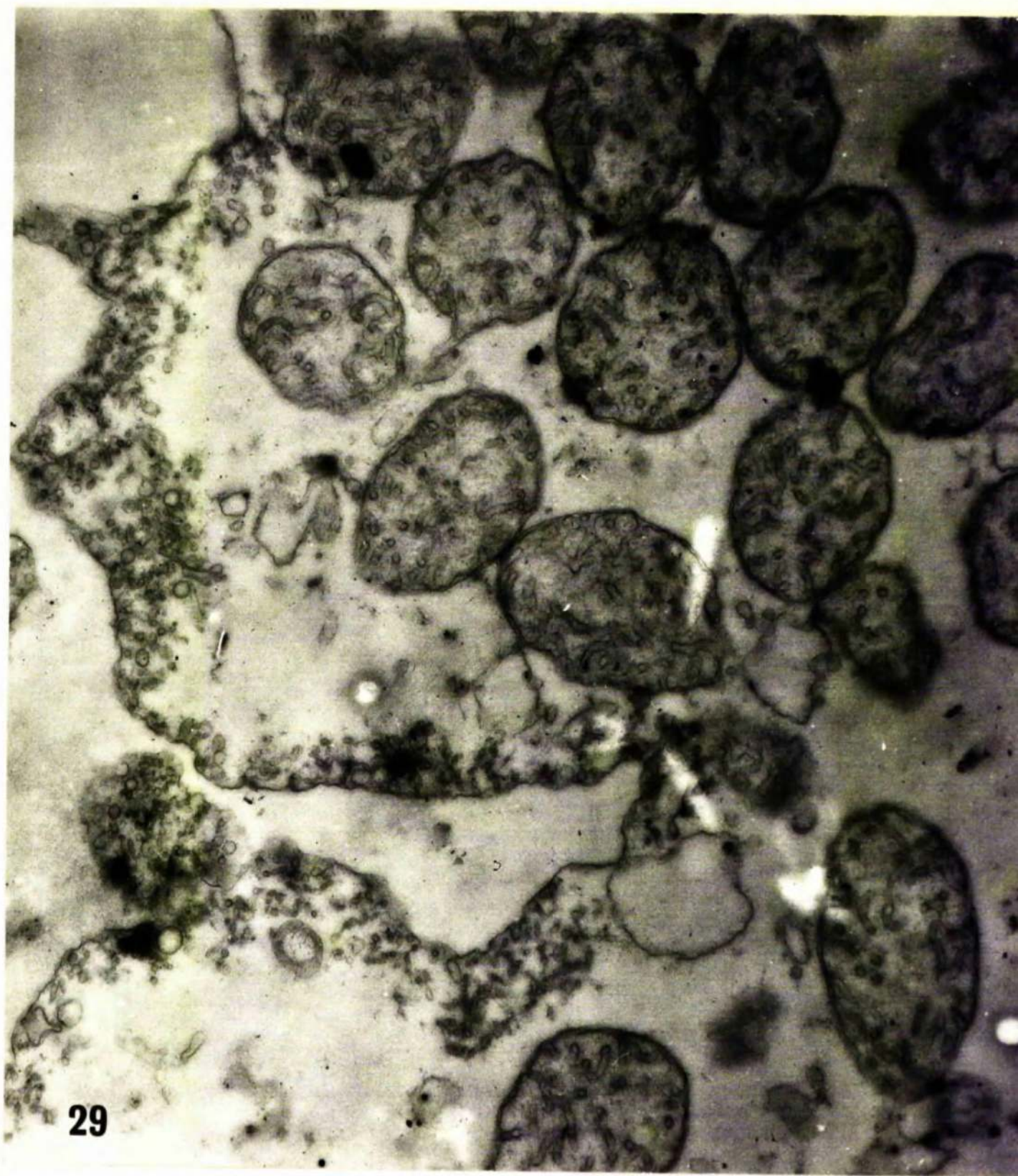


Micrograph 27 Cytomembranes of normal Amoeba
proteus. There is almost no trace of granules
on the surface without staining. (mag. 53,000).

Micrograph 28 After Karnovsky's stain the
cytomembranes have many 200 Å granules arranged
in whorls on the surface. There is little
difference in this case in general contrast.
(mag. 52,000).



Micrograph 29 Active contractile vacuole of
A. proteus. It is surrounded by microvesicles
some of which are in continuity with the wall
and by a thick layer of mitochondria. (mag. 15,000).

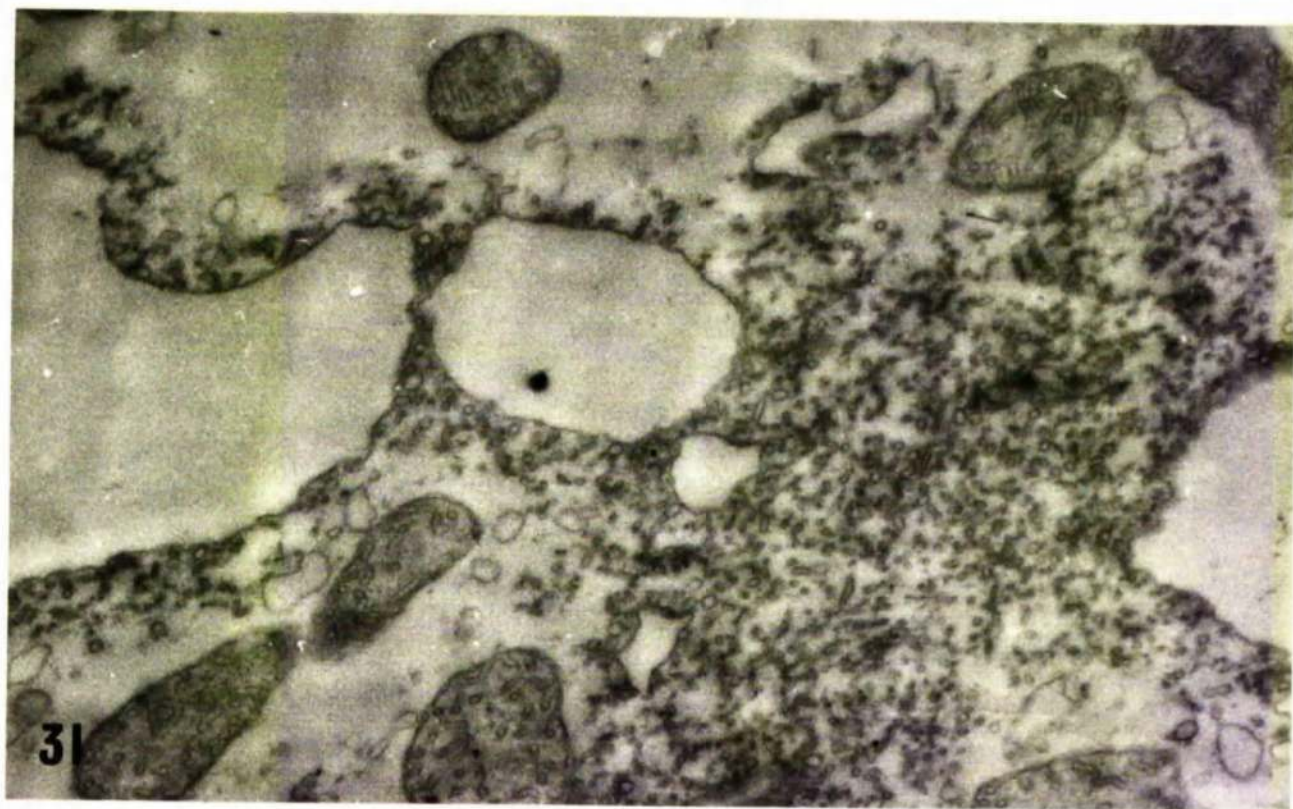
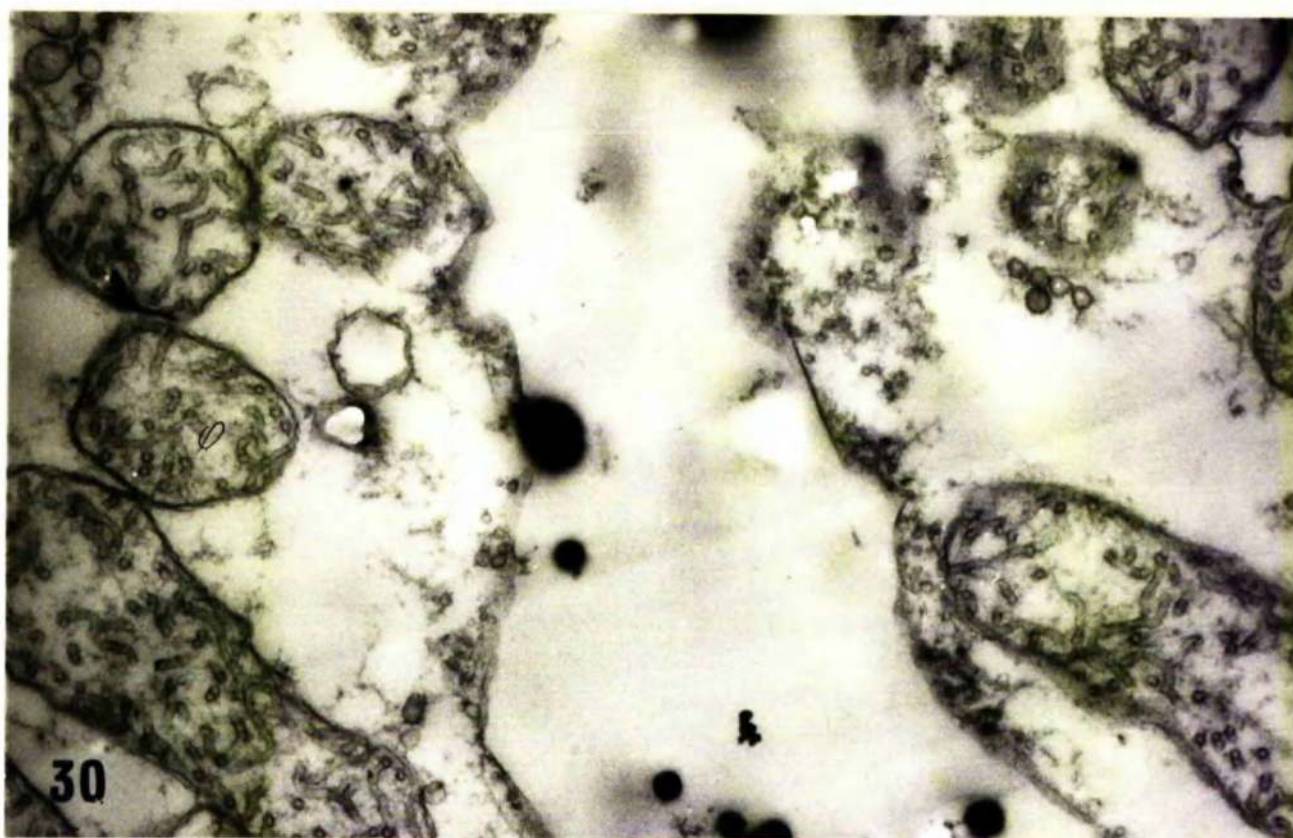


29

Micrographs 30 and 31 Part of the contractile vacuole of A. proteus. Micro 30 has sparse satellite vesicles round the periphery and a coat of mitochondria but 31 has many vesicles and is probably sectioned tangentially.

There is some dense debris in the lumen, mostly artefact and contamination on the sections.

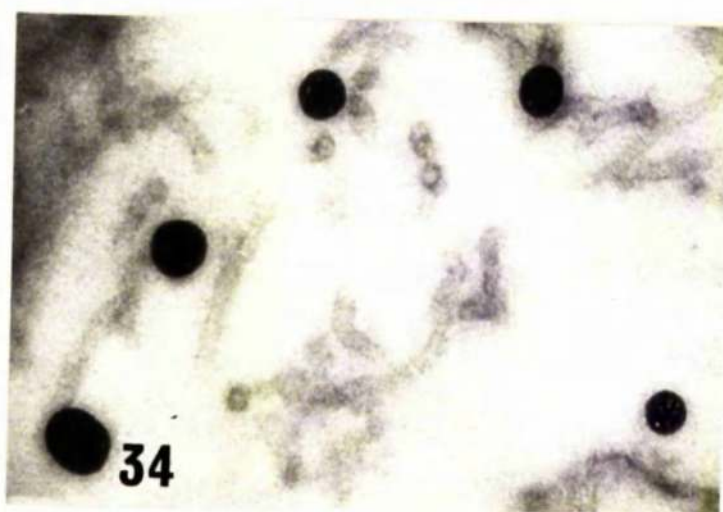
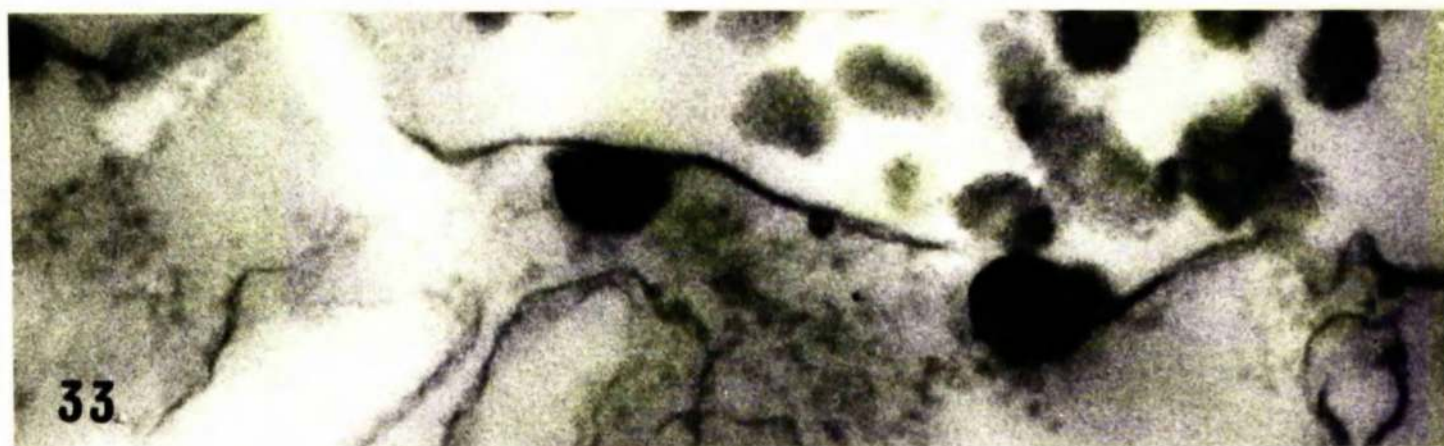
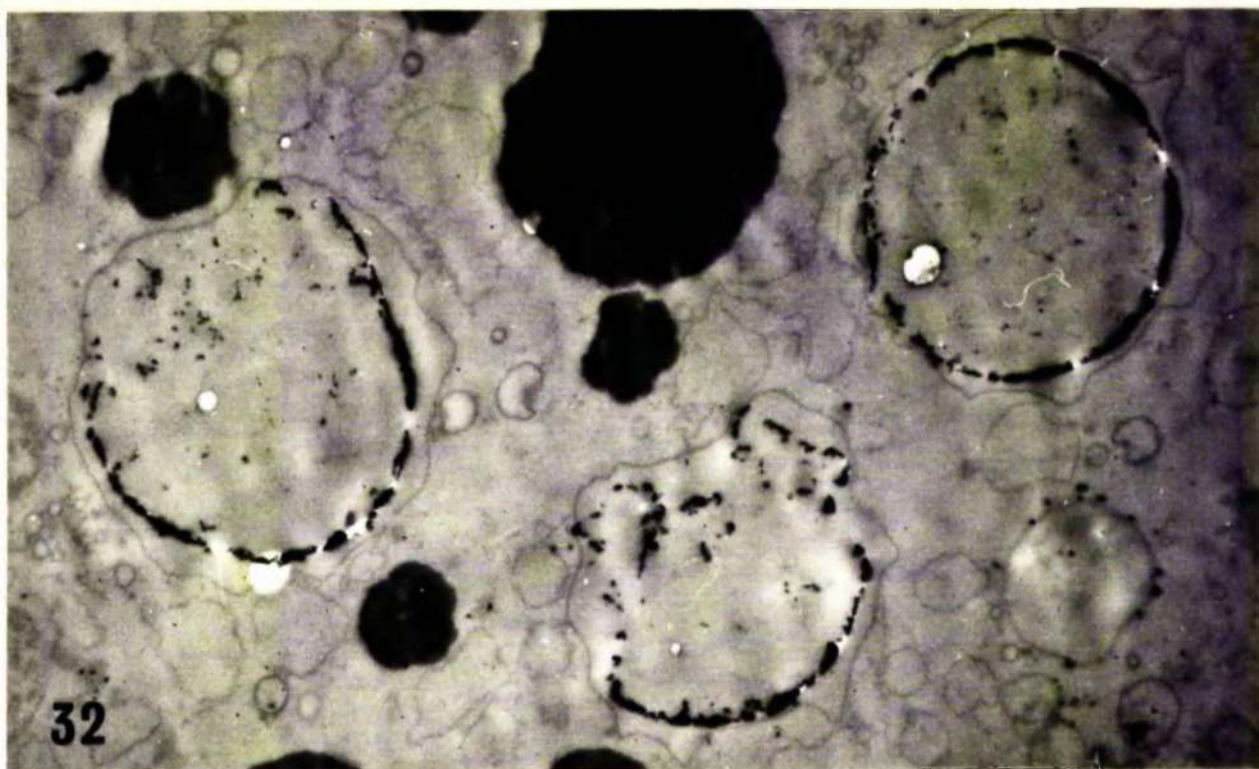
(mag. 30 = 13,000, 31 = 11,000).



Micrograph 32 Vacuoles in the cytoplasm of A. proteus. They are lined with a "shell" of small dense bodies connected by narrow dense filaments. In one case the dense shell is fragmented. The appearance suggests that dense granules attached to the cytoplasmic surface of the vacuoles might have become transferred into the lumen but the mechanism, if any, is completely obscure. (mag. 12,000).

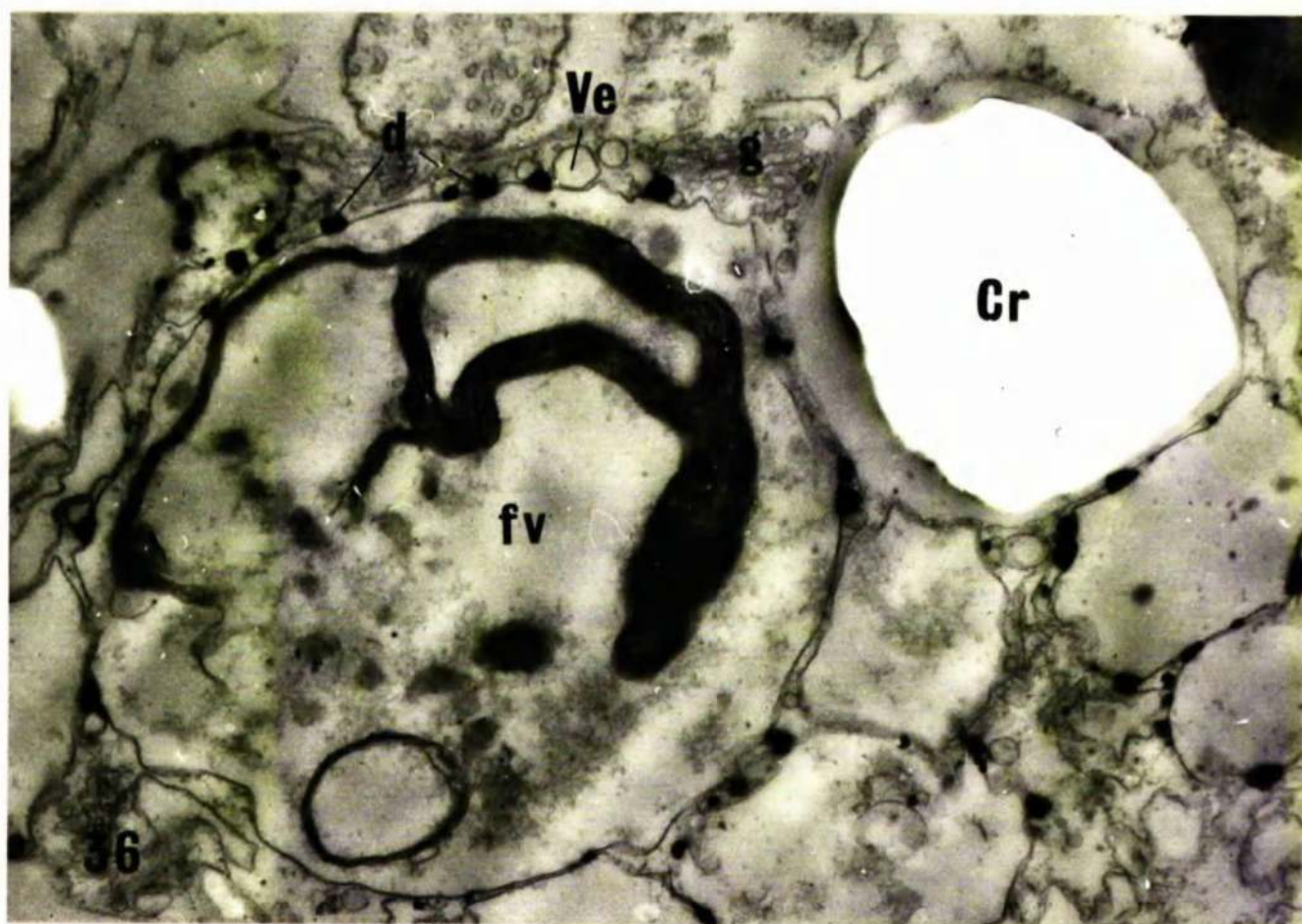
Micrograph 33 Small dense bodies on the cytoplasmic surface of a pinocytosis channel (resulting from alcian blue treatment); they are exactly similar to those normally occurring on the plasma membrane. There are small bubble-like internal translucencies. (mag. 76,000).

Micrograph 34 Small dense bodies lying free in the cytoplasm following potassium permanganate fixation. Their morphology and appearance is the same as following osmium tetroxide. (mag. 43,000).



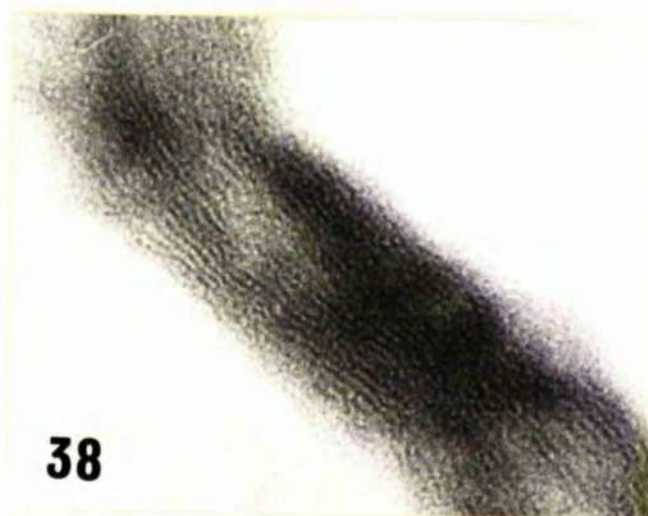
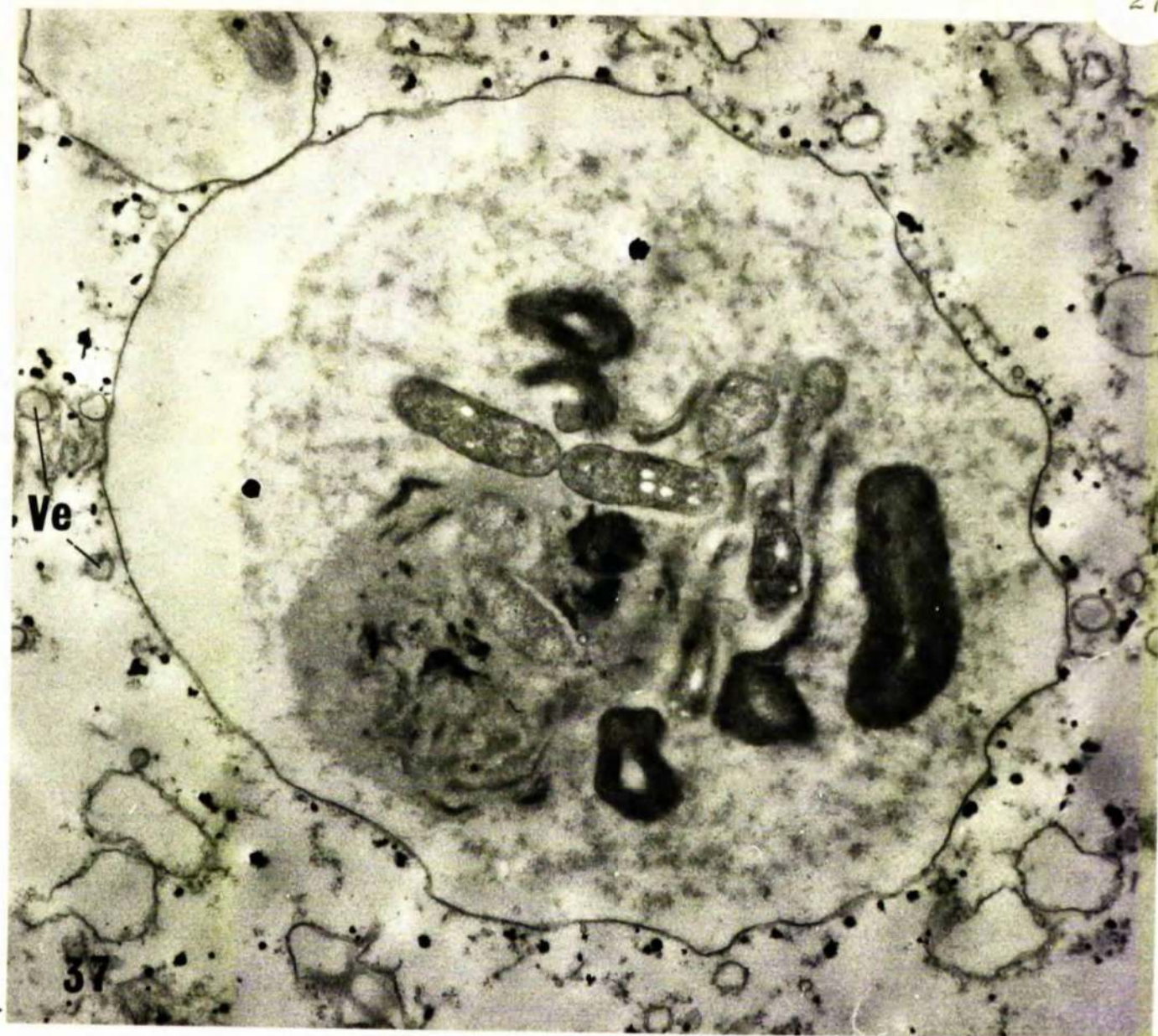
Micrograph 35 Food vacuole. The central material consists of three elements - flocculated granules, absent only from the periphery of the vacuole, lamellated masses rather like skeined wool and bacteria (b). The bacteria have translucent central lipid droplets. There are a few small dense bodies on the cytoplasmic surface of the food vacuole. (mag. 13,000).

Micrograph 36 A food vacuole (fv) containing well marked lamellated masses and a few patchy granular areas. A small Golgi apparatus (g) lies near the wall, as well as satellite vesicles (Ve). The food vacuole wall and some nearby cytoplasmic vesicles have small dense bodies on the cytoplasmic surface (d). Cr. - crystal negative, F - fat droplet. (mag. 15,000).

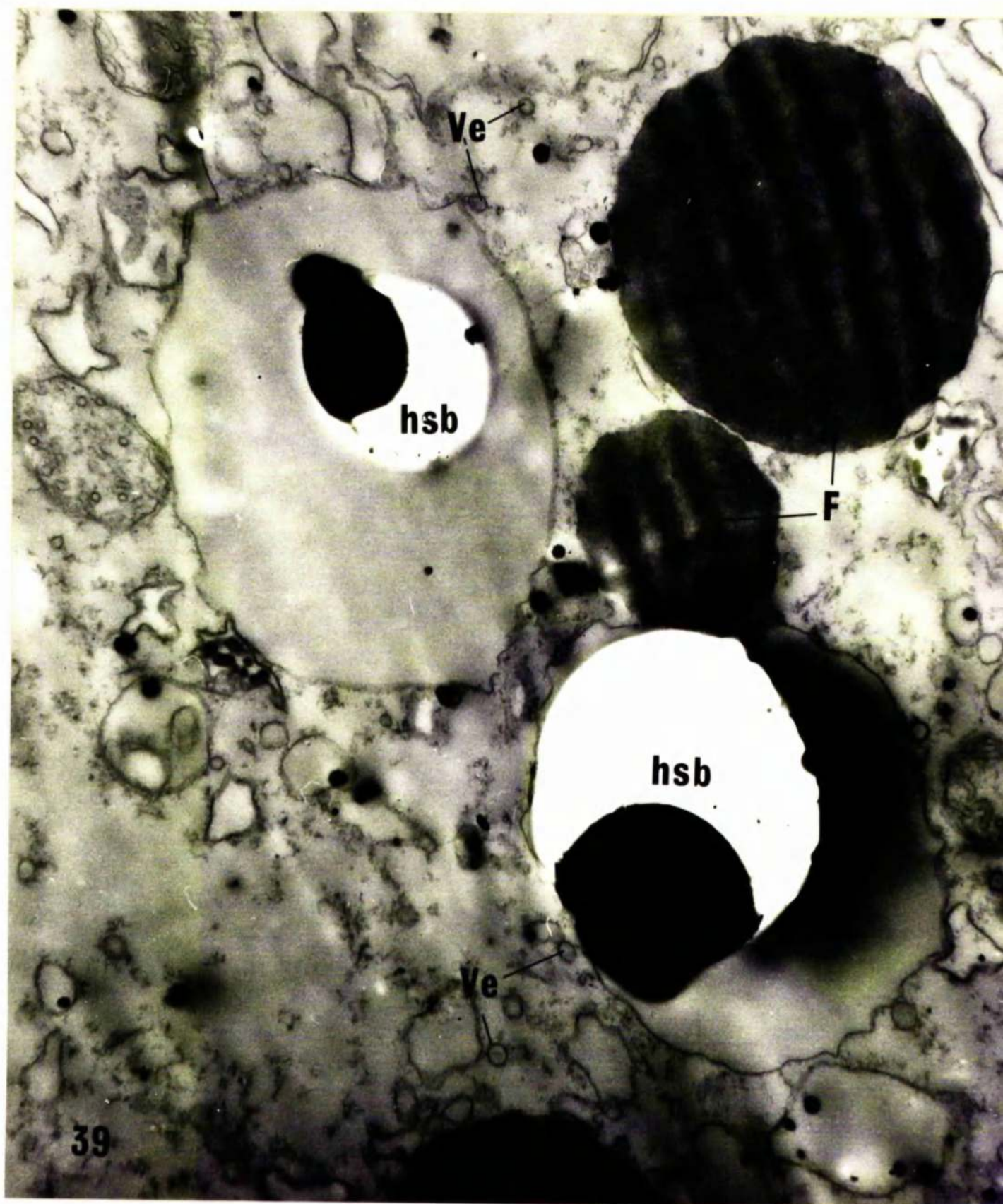


Micrograph 37 Food vacuole of Amoeba proteus containing bacteria, one of which is dividing, a granular ground substance and lamellated masses. There is some debris possibly food remains. The cytoplasm contains small round satellite vesicles (Vo). (mag. 17,500).

Micrograph 38 A higher magnification of lamellated mass of food vacuole of A. proteus. Each dense layer is approximately 40 - 50 Å wide and the intervening translucent layer a little wider. The explanation is considered in the text. (mag. 110,000).

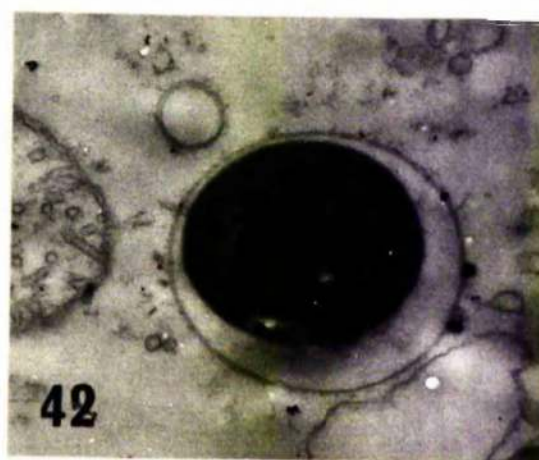
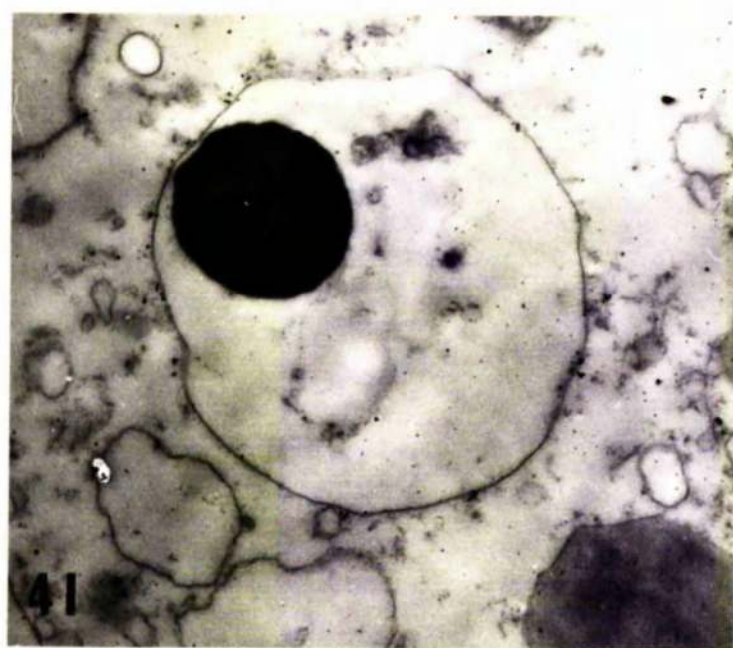
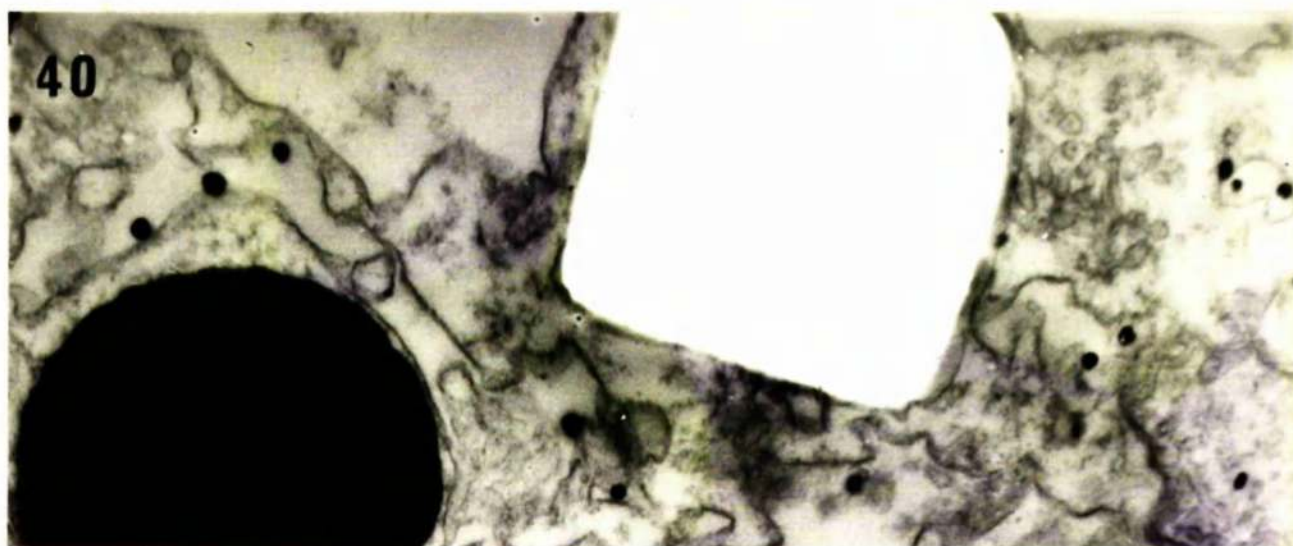


Micrograph 39 Heavy spherical bodies (hsb) with intensely dense cores and translucent halos. The fat droplets (F) show typical sectioning artefact. The cytoplasm contains small round vesicles (Ve). (mag. 17,000).

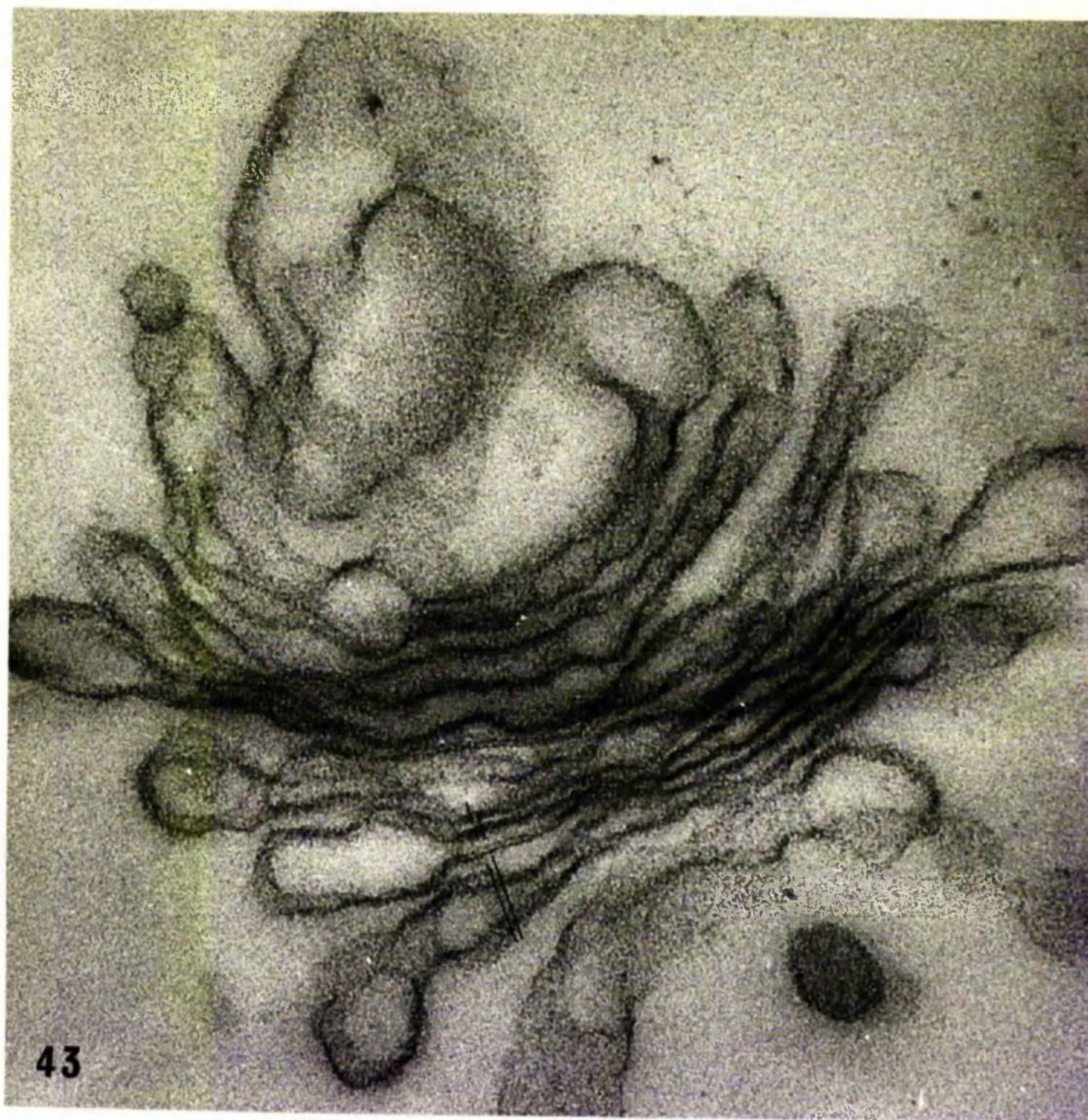


Micrograph 40 Crystal negative (Cr) and large
osmophilic fat droplets.

Micrographs 41 and 42 This type of structure is
occasionally found in normal organisms and
might be interpreted either as a fat droplet
within a membrane or a developmental stage of a
heavy spherical body. At present only the
position taken up after centrifugation is a
complete answer to the problem. No such structures
were seen in the heavy pole of centrifuged
Polomyxa.

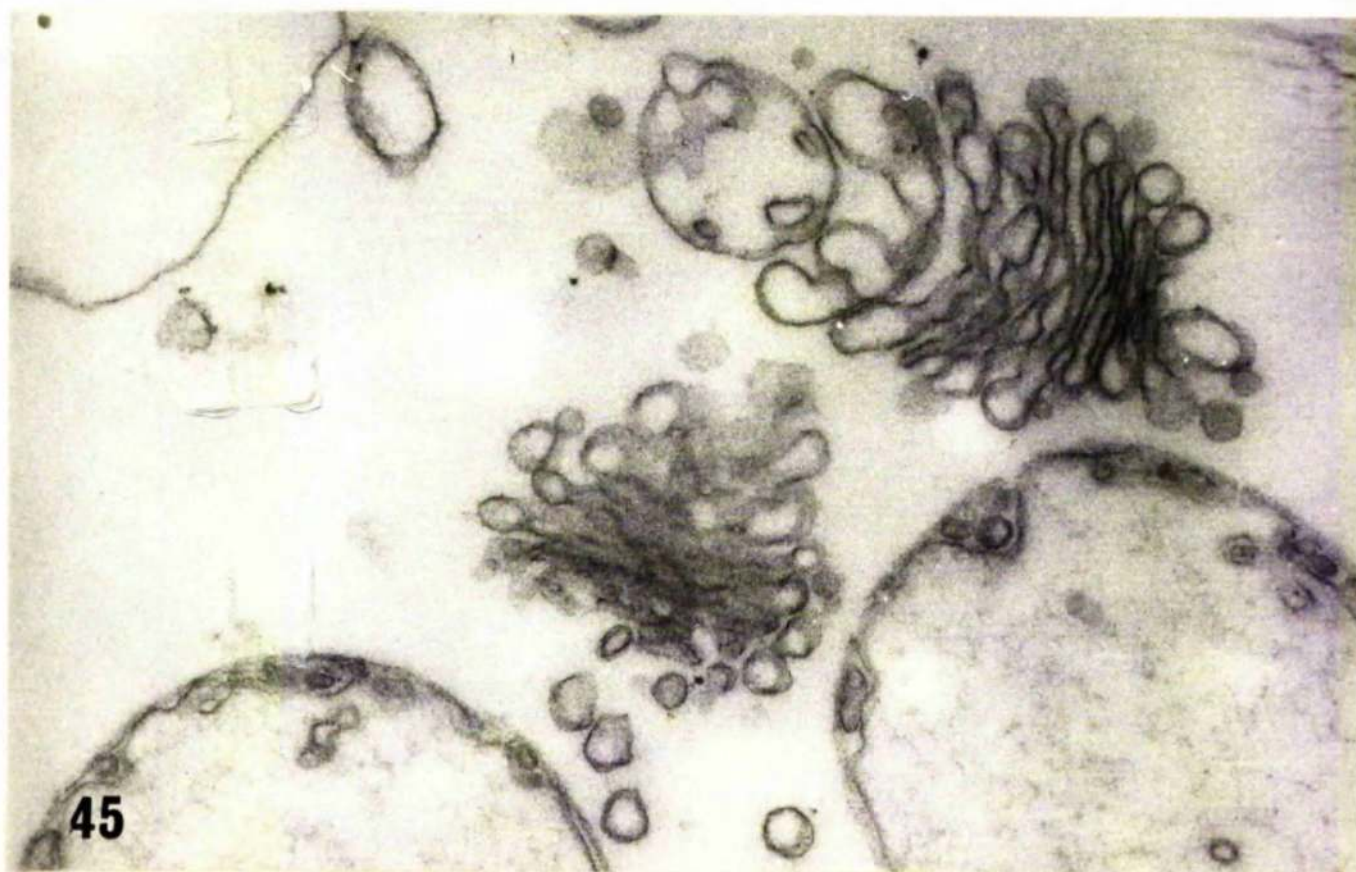
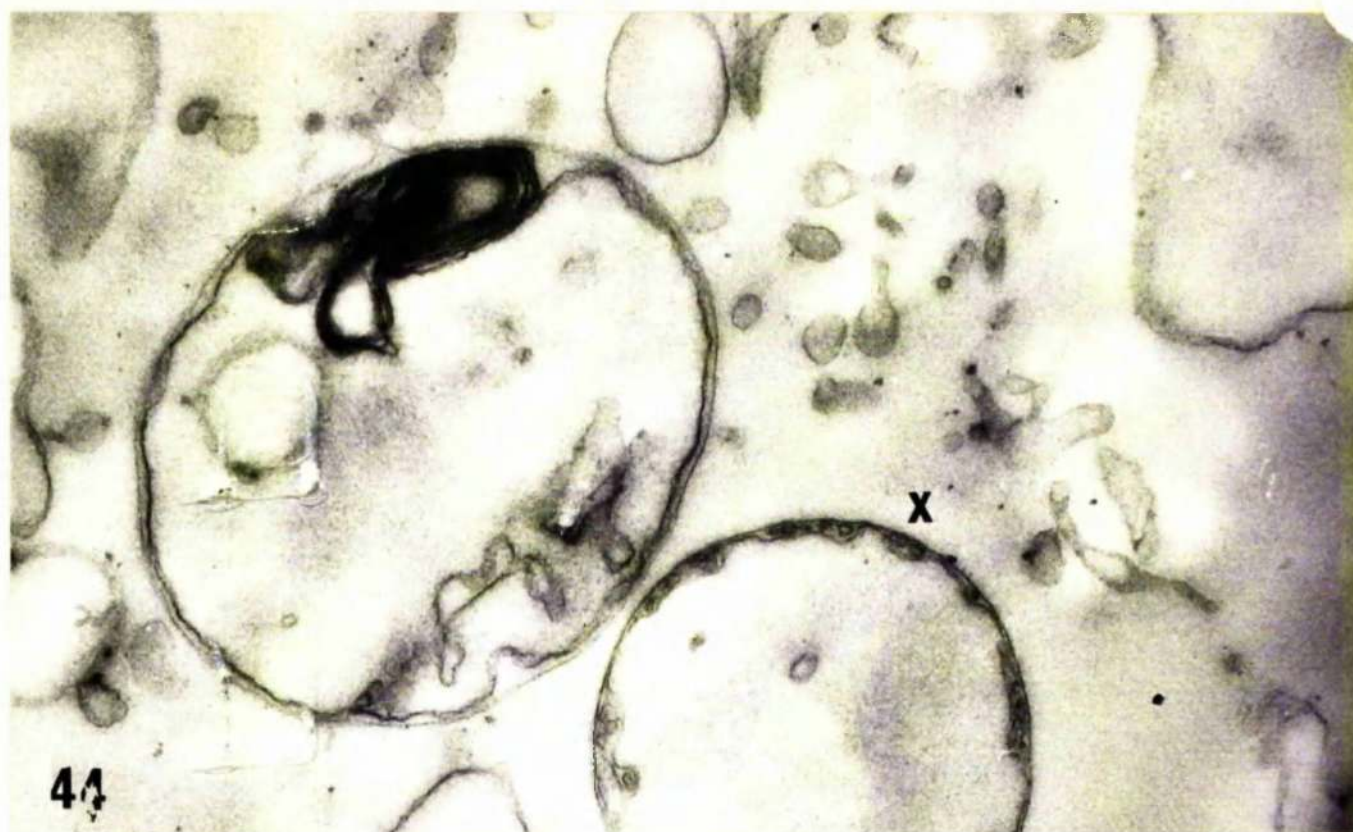


Micrograph 43 Well developed Golgi apparatus after permanganate fixation. The parallel membranes form the walls of flattened sacs and have been resolved to triple layering or unit membrane structure (= =). The periphery of the sacs appears to be dilated and the sacs become wider at the apex. It must be borne in mind that permanganate generally produces a smooth contouring (see micrographs 15 - 17). (mag. 125,000).



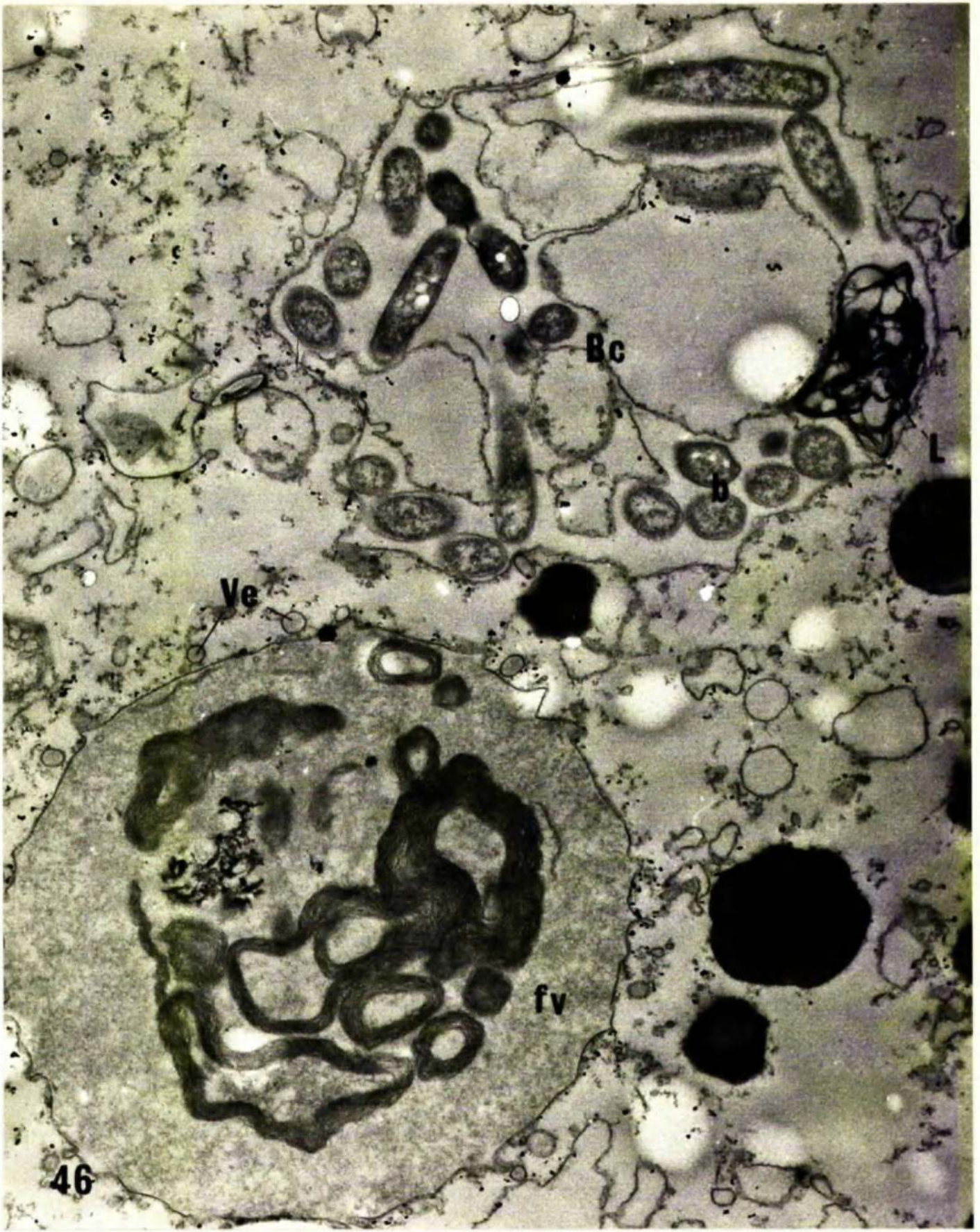
Micrograph 44 Mitochondria following potassium permanganate fixation. The right hand example shows almost complete absence of tubules and two outer membranes which are separated by round profiles. The outermost membrane is better defined and in places (X) is itself double - it may represent the pair of outer membranes of the mitochondrion. In the left hand example a large myelin figure has appeared between the two limiting membranes of which the inner in this case is the more dense. (mag. 31,000).

Micrograph 45 Golgi apparatus and mitochondria after permanganate. The mitochondria show the same pattern of preservation as in microg. 44 and the Golgi apparatus as 43.



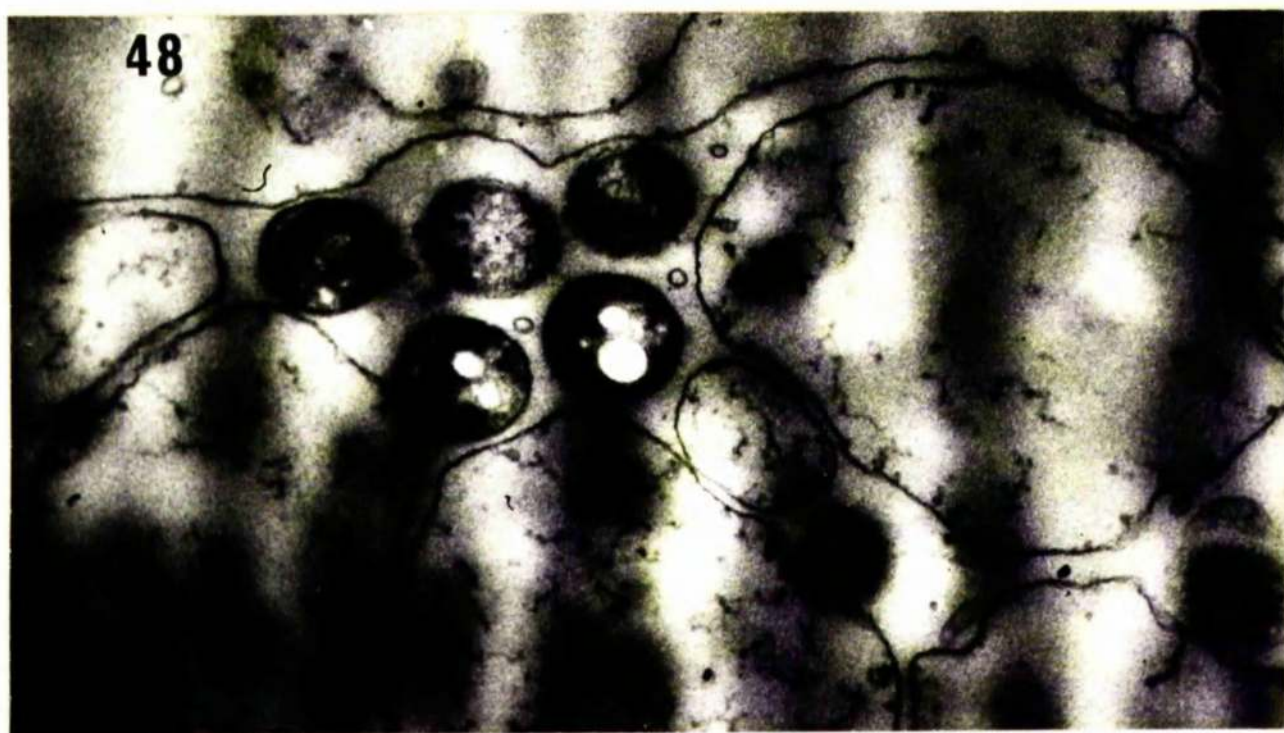
Micrograph 46 Cytoplasm from Amoeba proteus

Bristol strain showing a typical food vacuole (fv) and typical bacterial complex (bc). The food vacuole contains lamellated masses as well as a little dense debris in a granular ground substance. The wall is relatively thick and there are satellite vesicles (Ve). The bacterial complex contains bacteria (b) and inner vacuoles and one inner vacuole is occupied by a lamellated body (L). The wall is irregular and of low density comparable with the cytomembranes of surrounding cytoplasm. (mag. 35,000).



Micrograph 47 The simplest type of bacterial complex encountered. The irregular wall encloses only 4 bacteria and a number of small vesicles. (mag. 19,000).

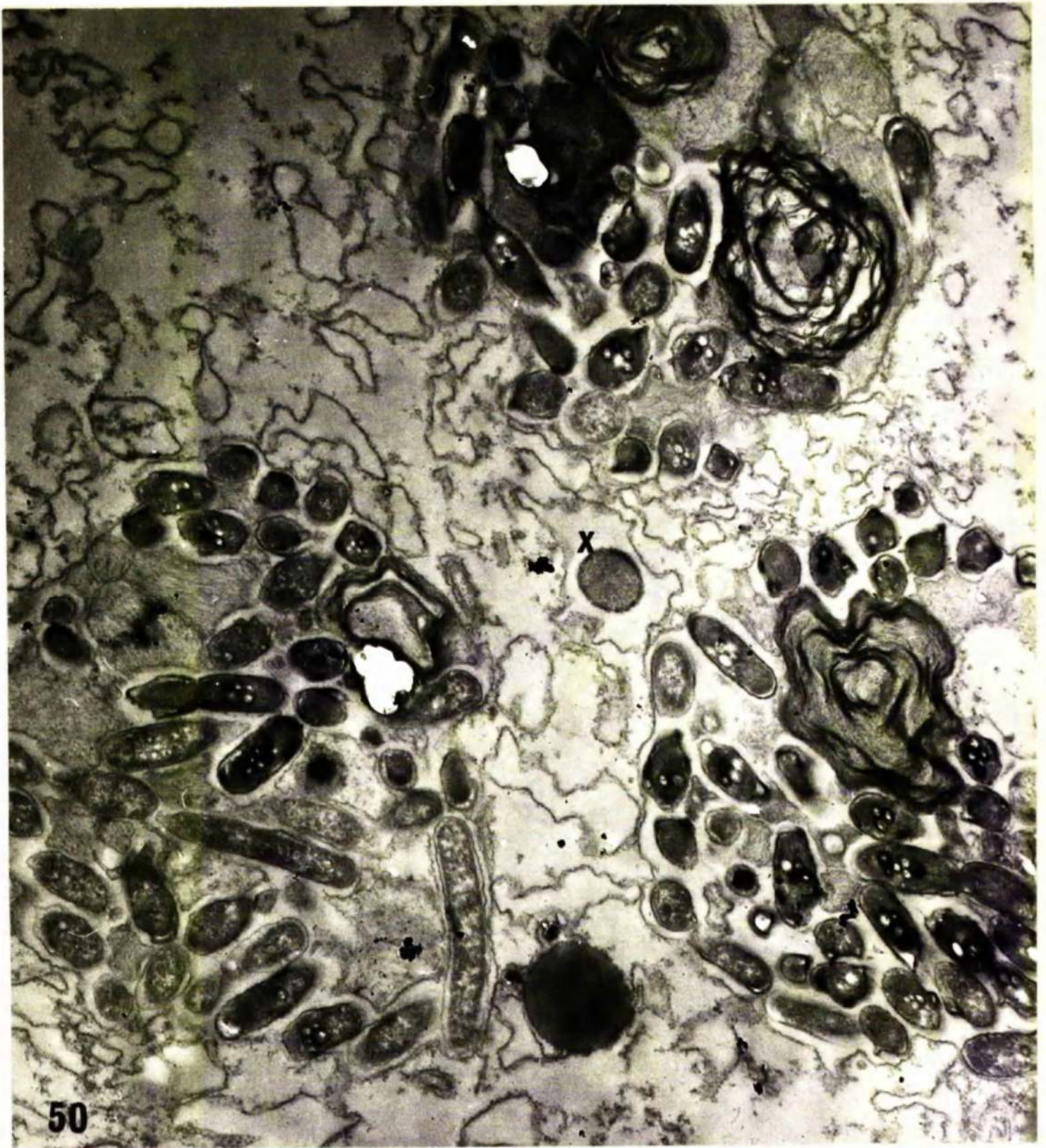
Micrograph 48 More complicated complex. Although a very poor section it shows the independence of inner vacuole walls and outer walls. The bacteria contain lipid droplets like the bacteria of the food vacuole. (microg. 35). (mag. 25,000).



Micrograph 49 Large bacterial complex showing the usual features. Some of the inner vacuoles contain dense packed fibrils but others are empty. The bacteria are not in contact with each other or the complex wall. There is a lamellated body of high electron density. (mag. 12,000).



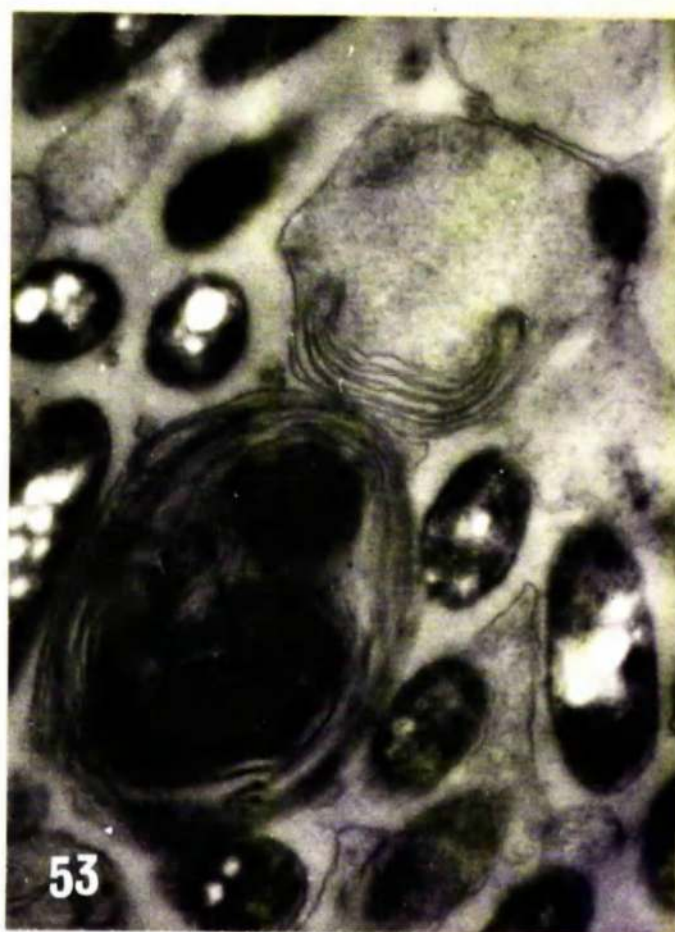
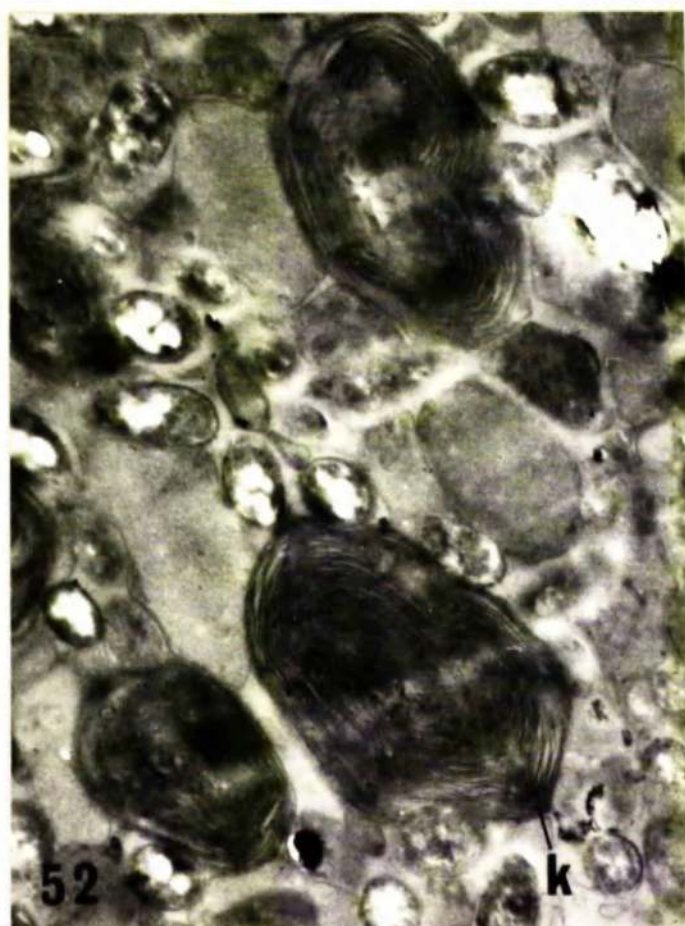
Micrograph 50 Bacterial complexes after PTA staining. The outlines of lipid droplets in the bacteria are intensely stained and the bacterial wall relatively denser in some examples. The granulation in the cytomembranes is visible but like the cytomembranes themselves, the outer wall of the complex is not specifically intensified. In the centre (X) appears to be a fat droplet enclosed by cytomembranes. (mag. 17,000).



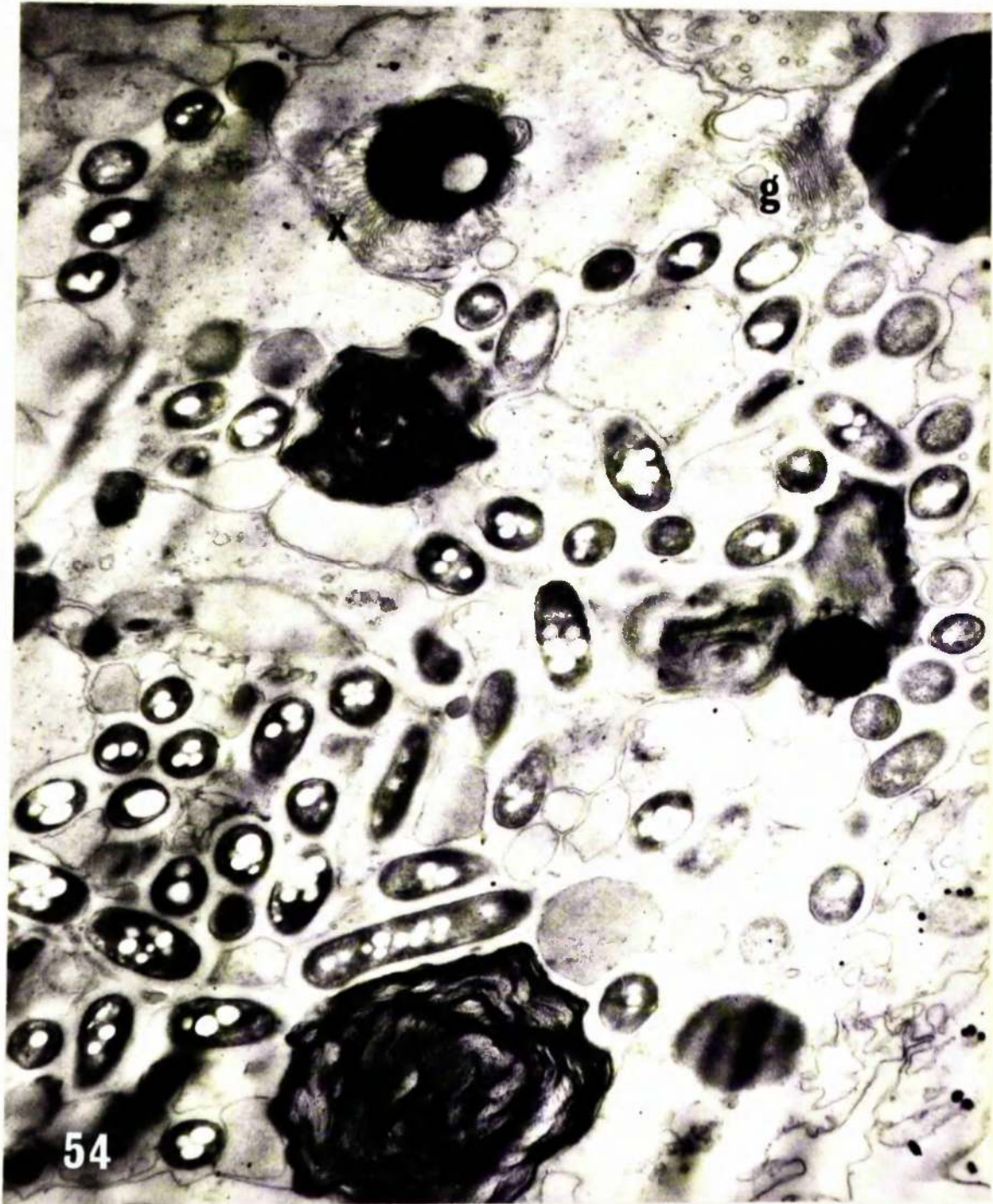
Micrograph 51 Part of typical bacterial complex
with a partially lamellated inner vacuole.
(mag. 14,500).

Micrograph 52 Lamellated bodies. One has a
knot-like structure with fusion of neighbouring
lamellae (k). (mag. 14,000).

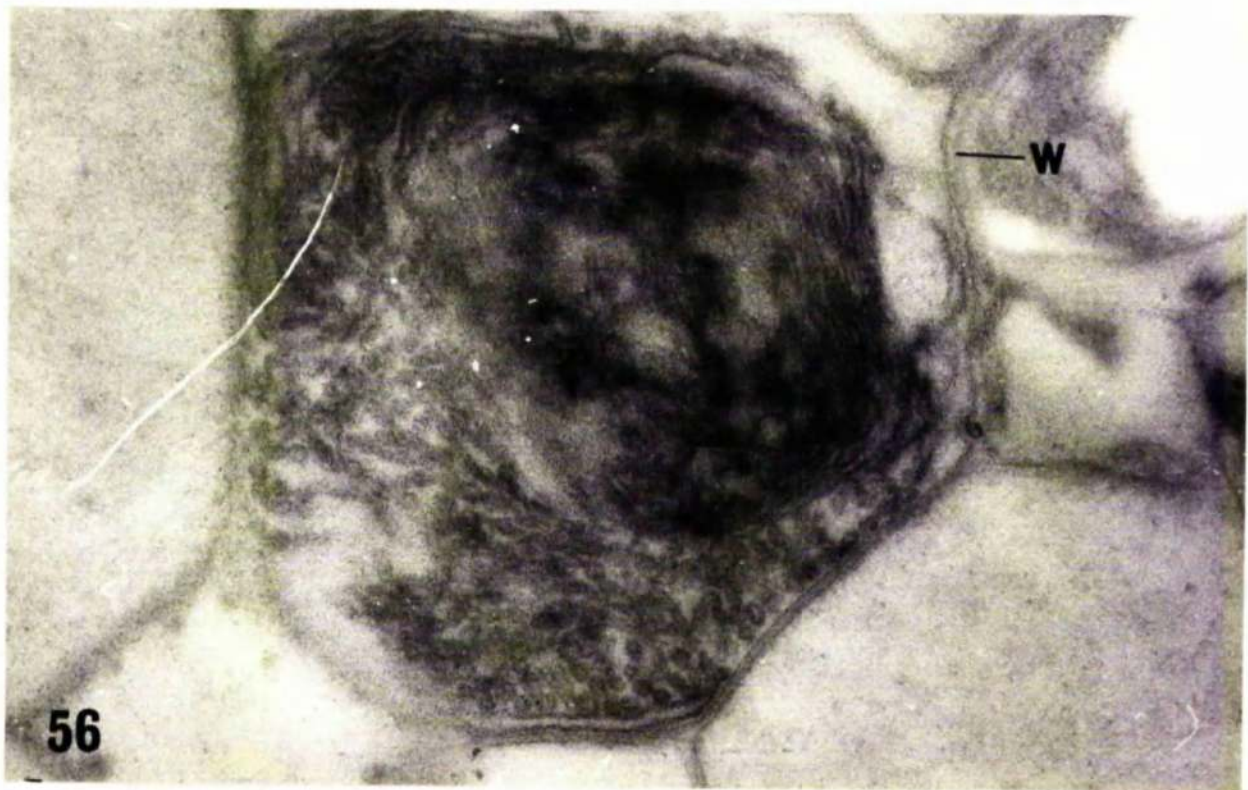
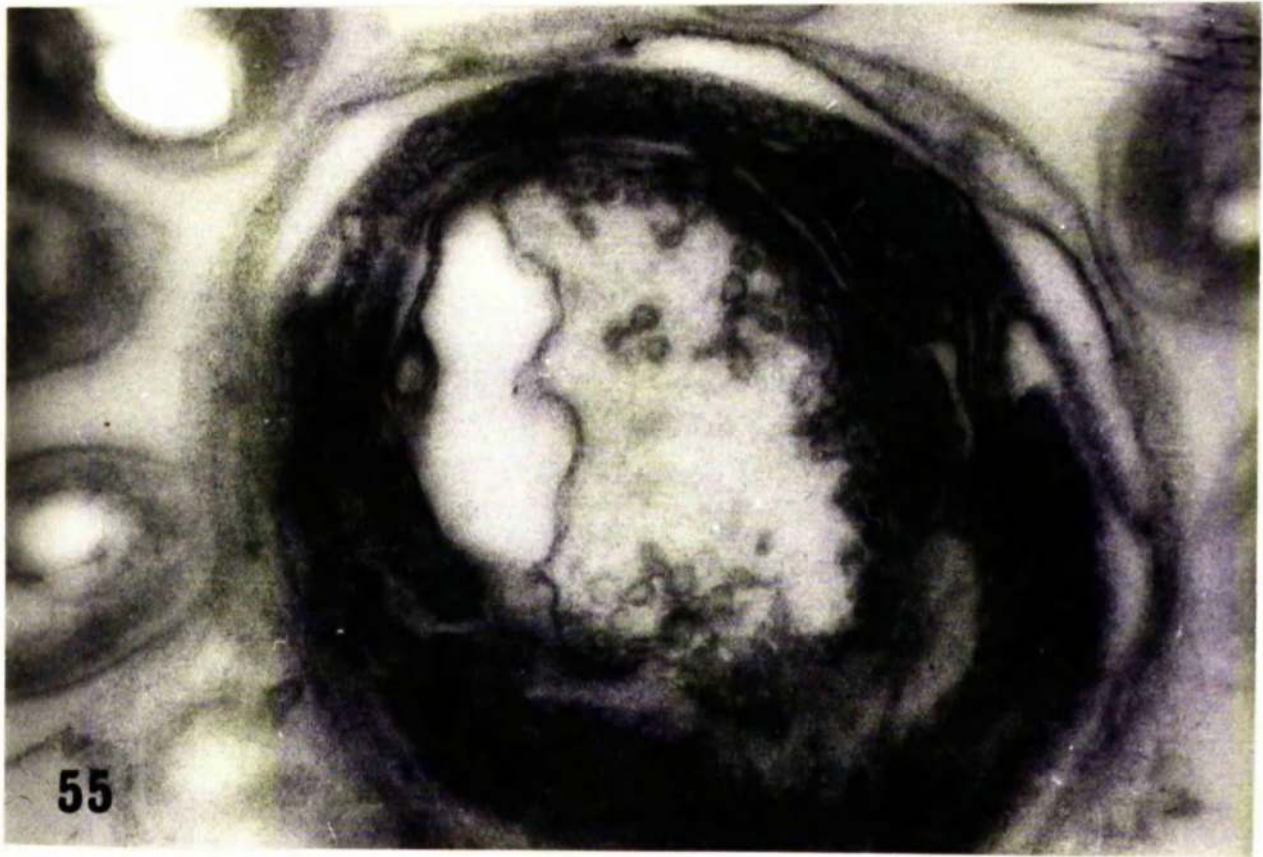
Micrograph 53 Lamellated bodies. One is complete
and is based on two centres, probably the result
of sectioning a kidney shaped mass and the other
has peripheral lamellae arranged like the Golgi
membranes as flat, curved sacs. (mag. 29,000).



Micrograph 54 In this bacterial complex an osmophilic mass has radiating membranes reminiscent of Golgi material (X). A normal Golgi apparatus as well as a mitochondrion and a fat droplet are outside the complex. (top right). (mag. 15,000).



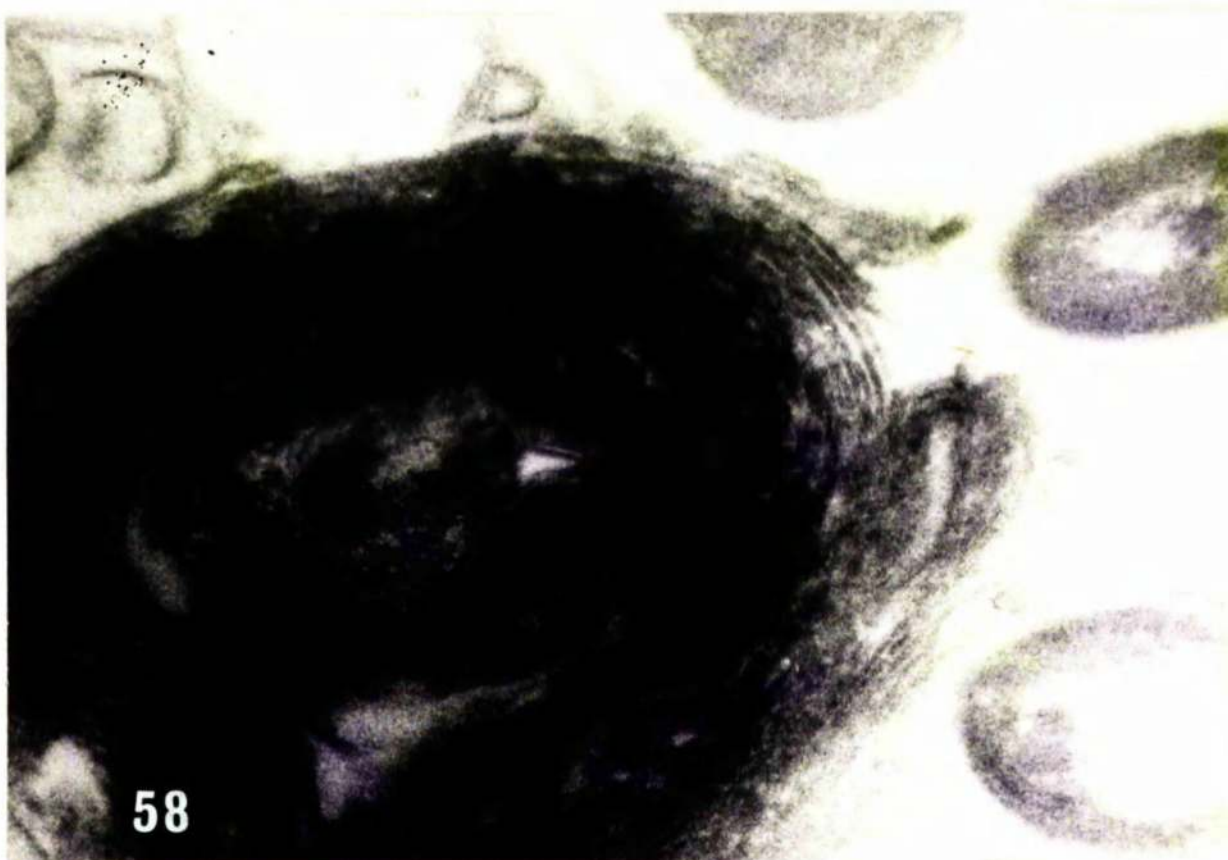
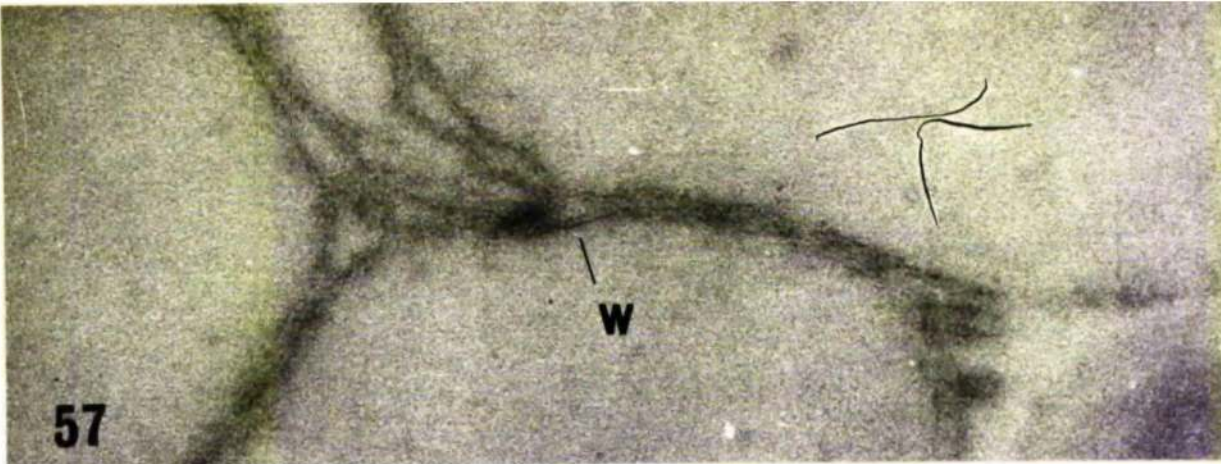
Micrographs 55 and 56 Lamellated bodies after permanganate fixation. The lamellae are accompanied by tubular outlines either centrally (55) or peripherally (56). Some of the lamellae are resolved into triple layering as well as the inner vacuole wall (W). (mag. 55 - 70,000, 56 - 57,000).



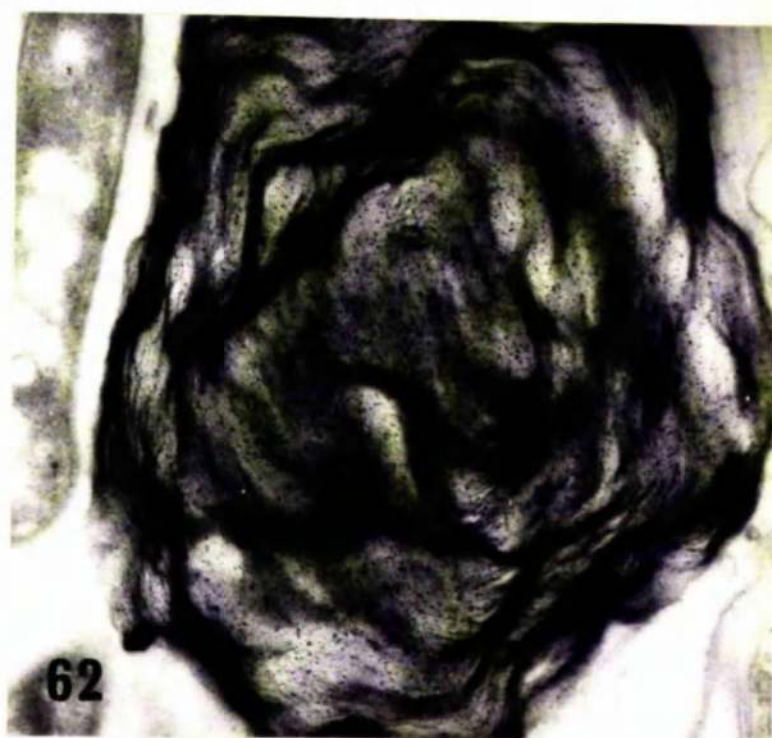
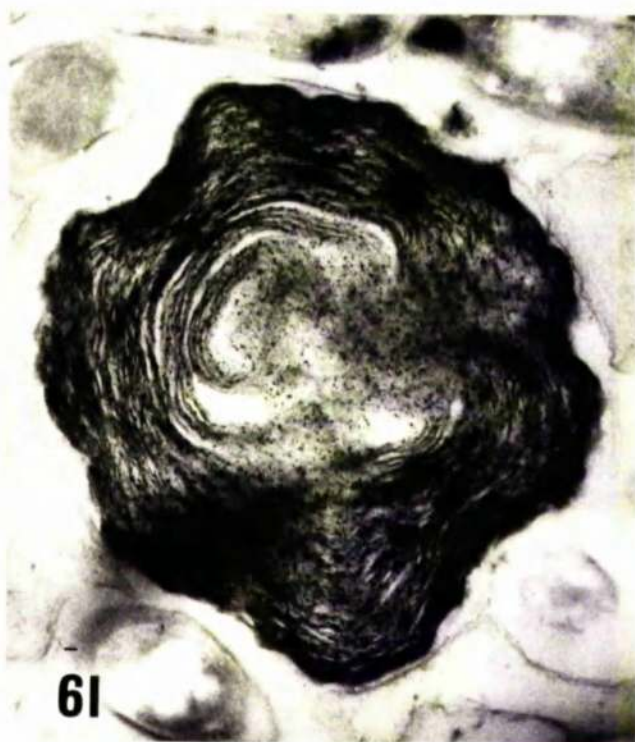
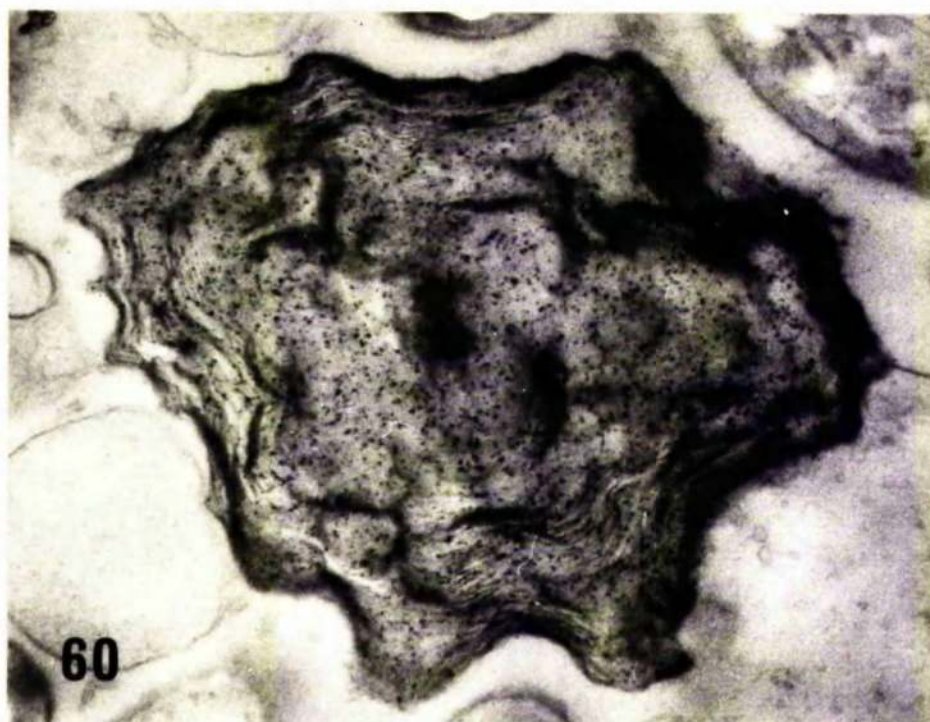
Micrograph 57 Higher magnification of the inner vacuole walls showing a triple layered or unit membrane structure (W) after permanganate fixation. (mag. 120,000).

Micrograph 58 A permanganate fixed lamellar body with triple layering clearly resolved in the lamellae. (mag. 77,000).

Micrograph 59 Part of 58 with higher optical enlargement. (mag. approx. 120,000).

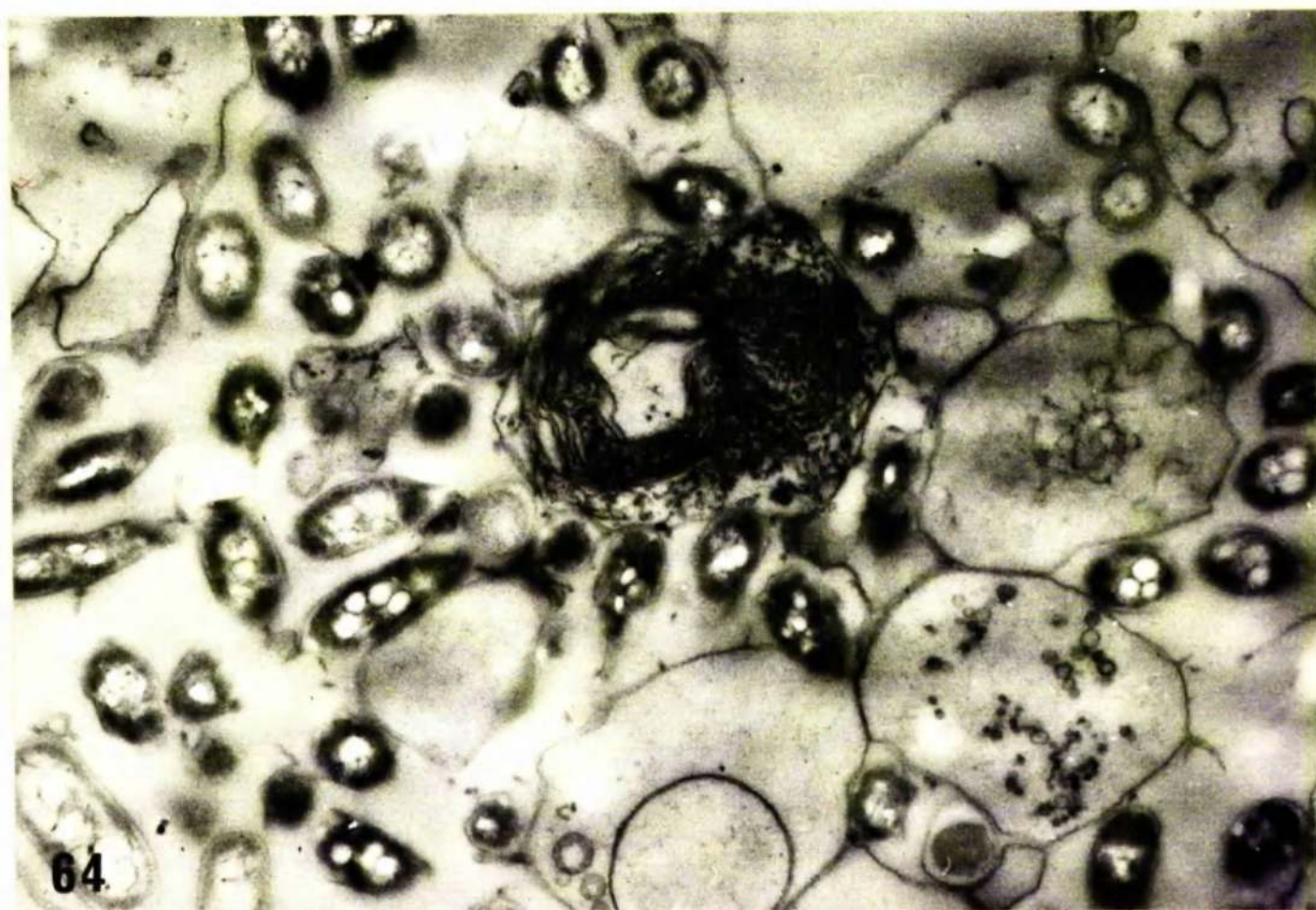
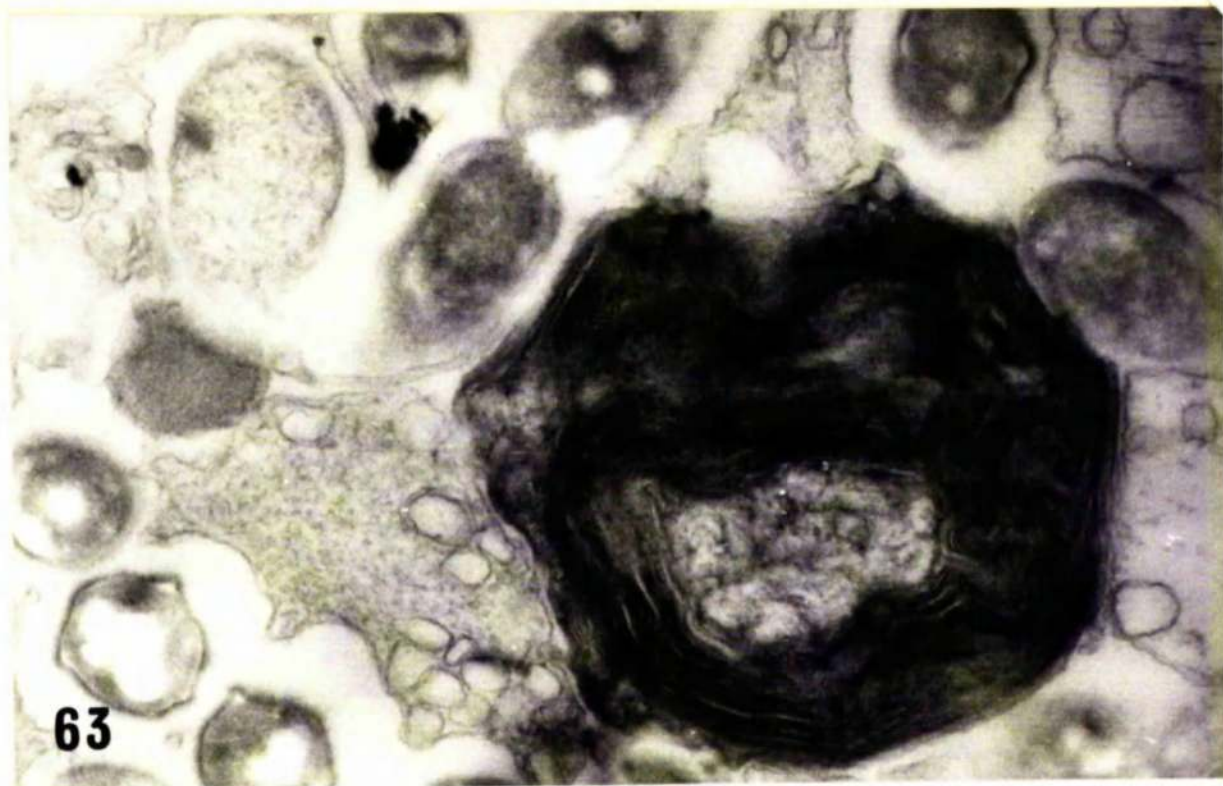


Micrographs 60 - 62 Various forms taken up by lamellated bodies after osmium fixation but never seen after permanganate. The lamellae appear to have collapsed in many places and are accompanied by small dense granules not found elsewhere in the complex. (mags. 60 - 33,000, 61 - 29,000, 62 - 27,500).



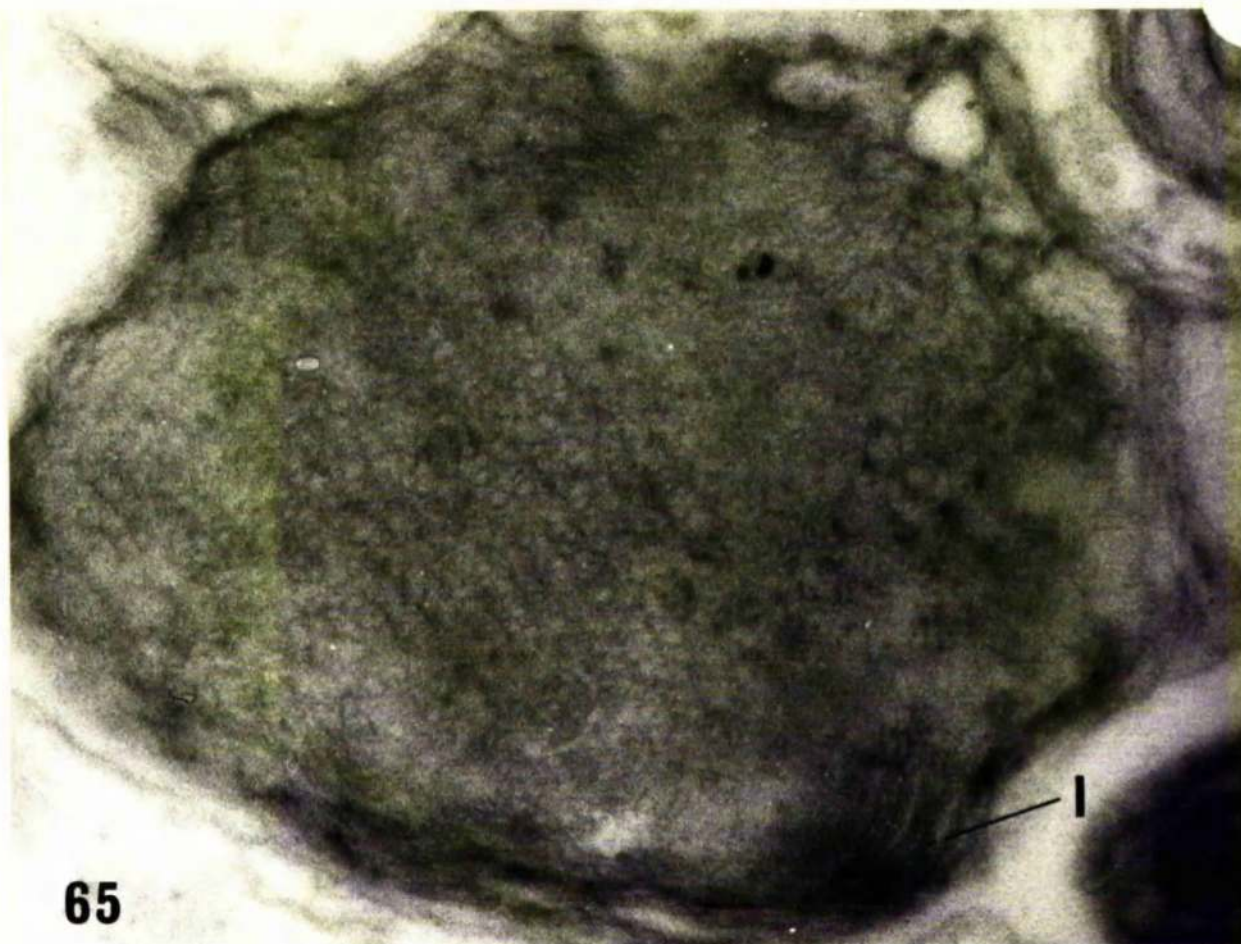
Micrograph 63. Lamellated body of bacterial complex from the same section as micrograph 49. Parts of the osmiophilic mass are distinctly lamellated but the regular pattern is broken down elsewhere, possibly because of the plane of sectioning. The centre has a soap-bubble appearance and there are small dense granules overall. (mag. 34,000).

Micrograph 64. Permanganate fixed specimen showing the intensification of membranes seen in this technique and the tubular structures present in the lamellated mass. There are no dense granules. (mag. 22,000).



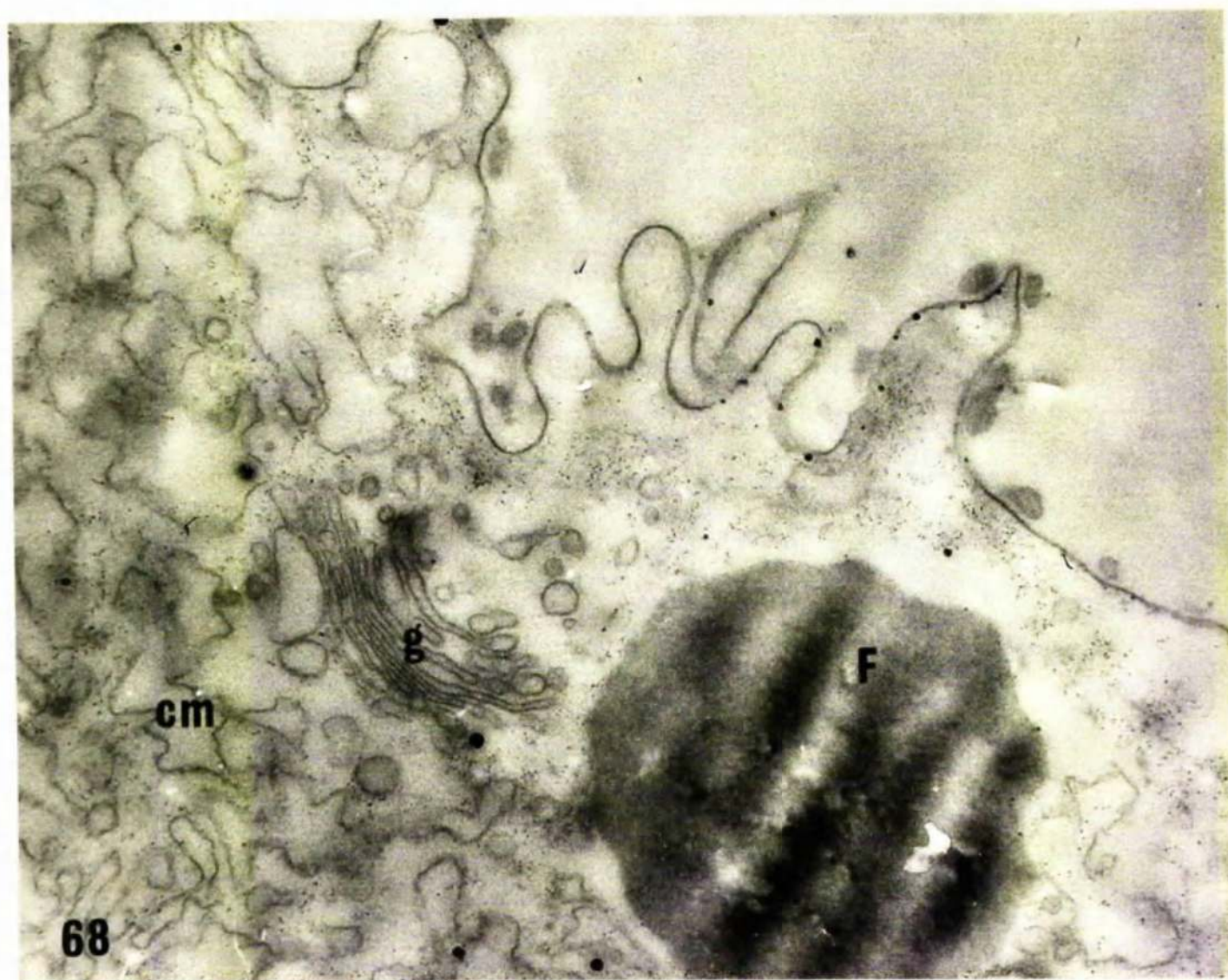
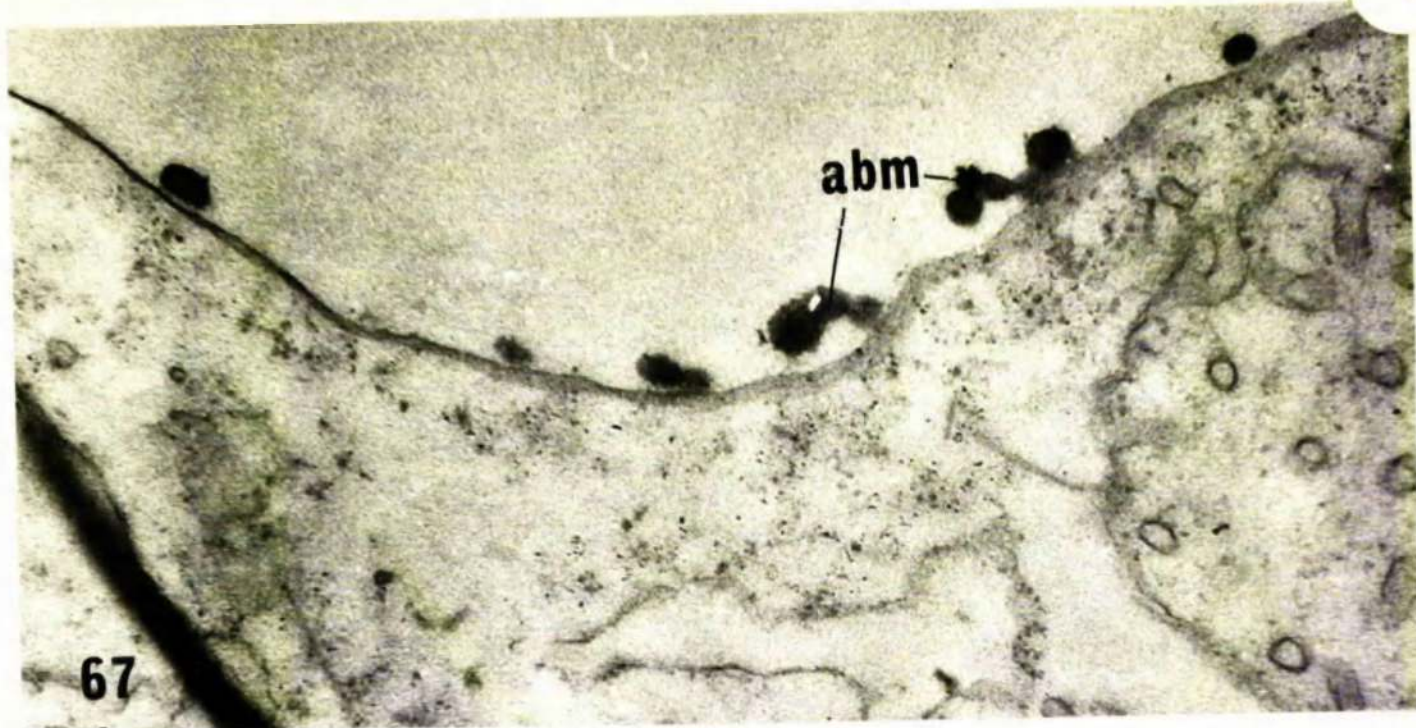
Micrograph 65. An inner vacuole of this specimen is occupied by a large, moderately osmiophilic mass. The background has a suggestion of a regular pattern but here and there lamellae are formed (1). (mag. 78,000).

Micrograph 66. A similar mass in a different specimen. The background material is reticulated and lamellae are seen only in part of the substance (1). (mag. 22,000).



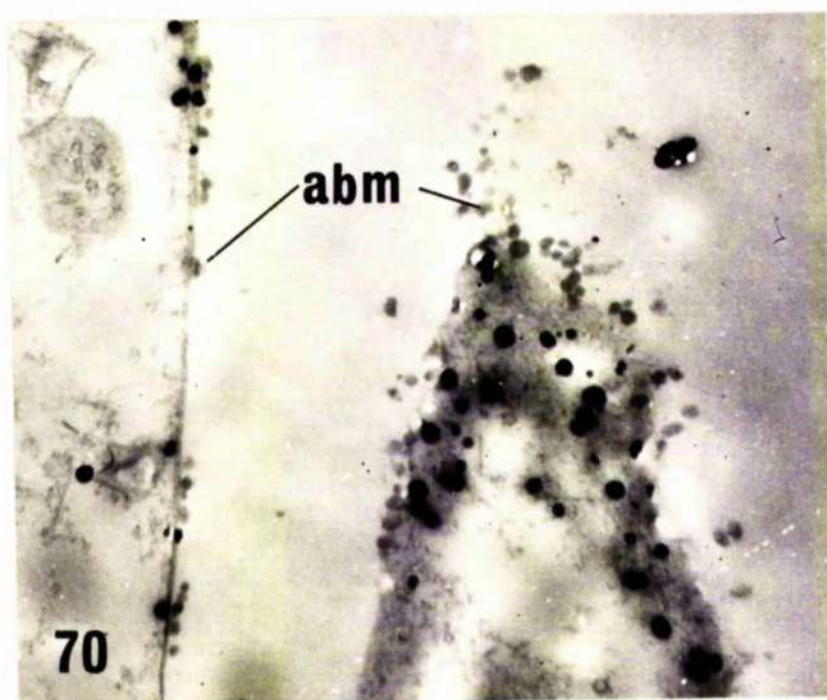
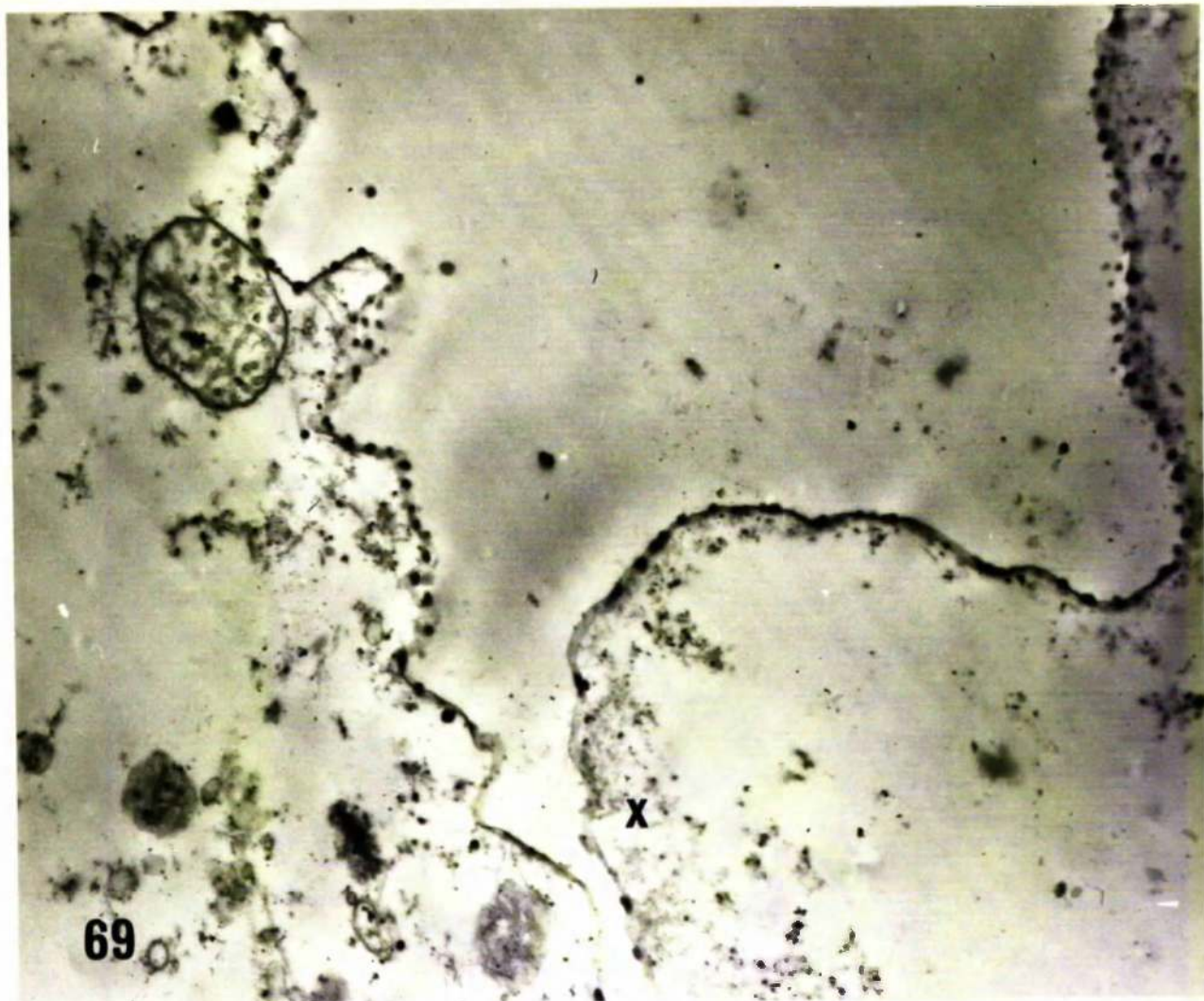
Micrograph 67 The plasmalemma of Amoeba proteus two minutes after immersion in alcian blue solution. The position of the filamentous layer is taken by dense rounded masses of alcian-blue-mucoid complex (abm). The underlying cytoplasm shows coarse granulation but contains normal cell constituents. (mag. 40,000).

Micrograph 68 Five minutes after alcian blue the granulation in the cytoplasm still containing normal cell constituents (g - Golgi zone, F - fat droplet, cm - cytomembranes) is accompanied by pleating of the plasmalemma which is interpreted as an incipient pinocytosis channel. (mag. 28,000).

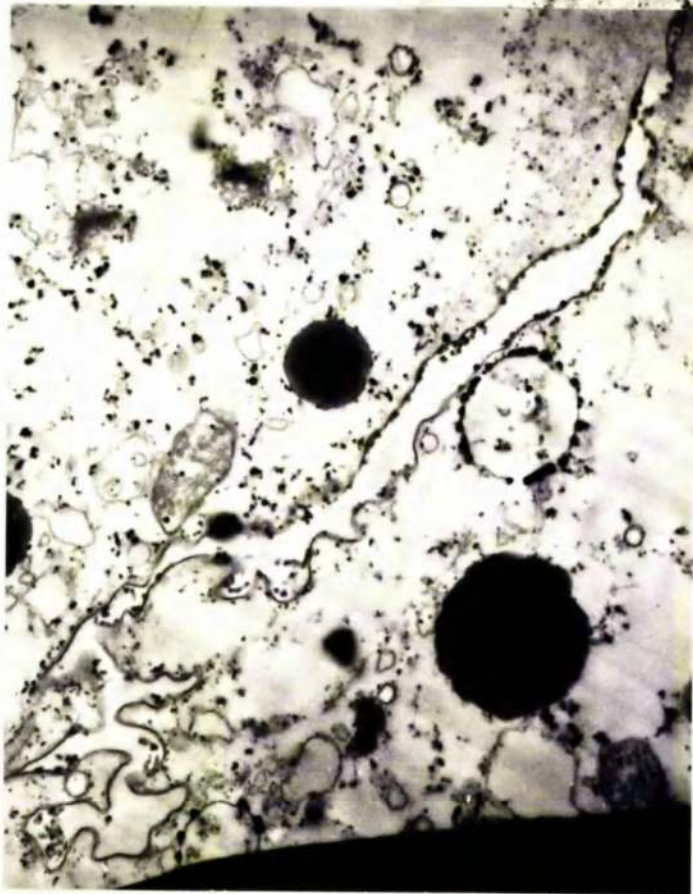
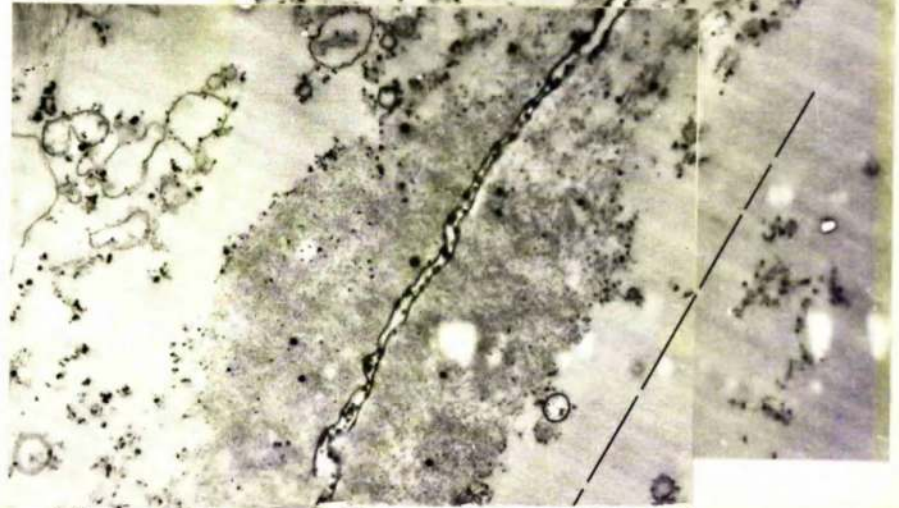
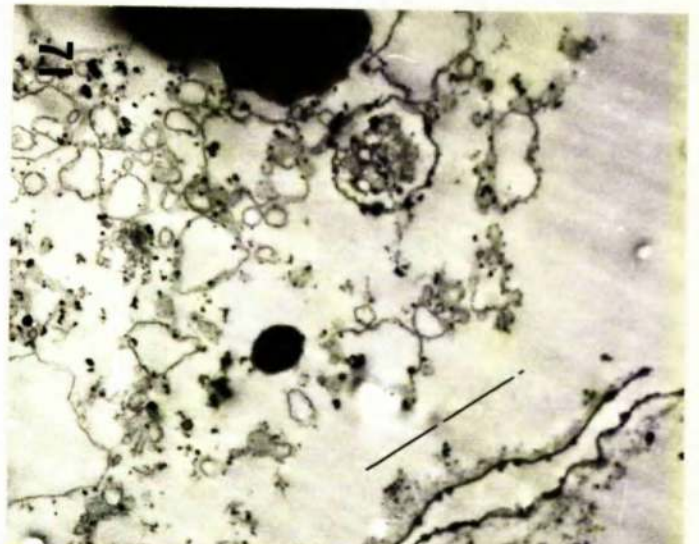


Micrograph 69 Mouth of an alcian blue channel
ten minutes after immersion. Much of the
alcian blue mucoid has separated from the
plasmalemma. There is one mitochondrion near
the mouth and the cytoplasm is granular for a
short depth. There is a large quantity of
small dense bodies on the cytoplasmic surface
of the membrane. (mag. 9,000).

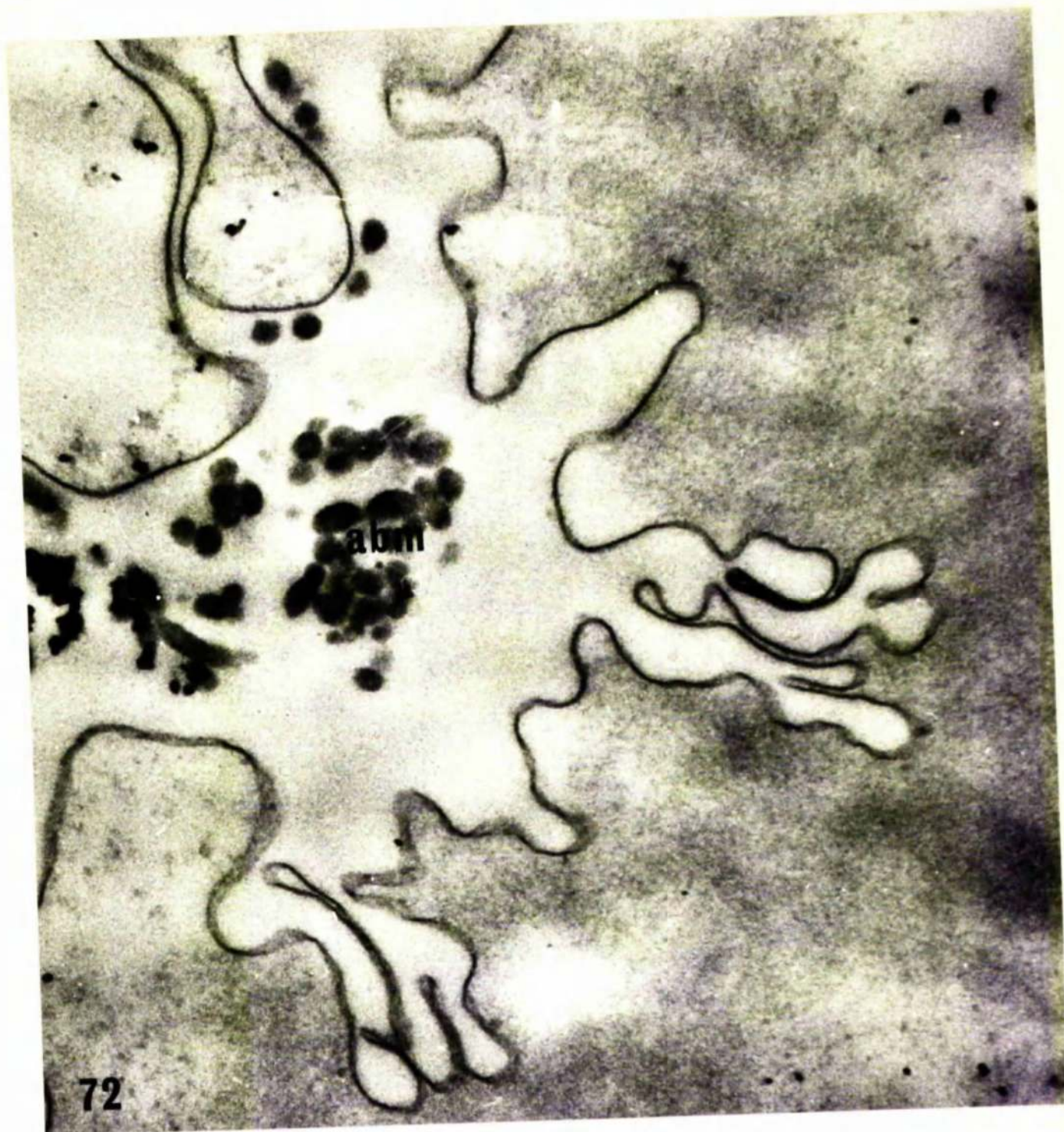
Micrograph 70 Tangential section of the plasmalemma
showing the relationship of abm and small dense
bodies. The former lying randomly over the outer
surface and the latter similarly arranged inside.
(mag. 11,500).



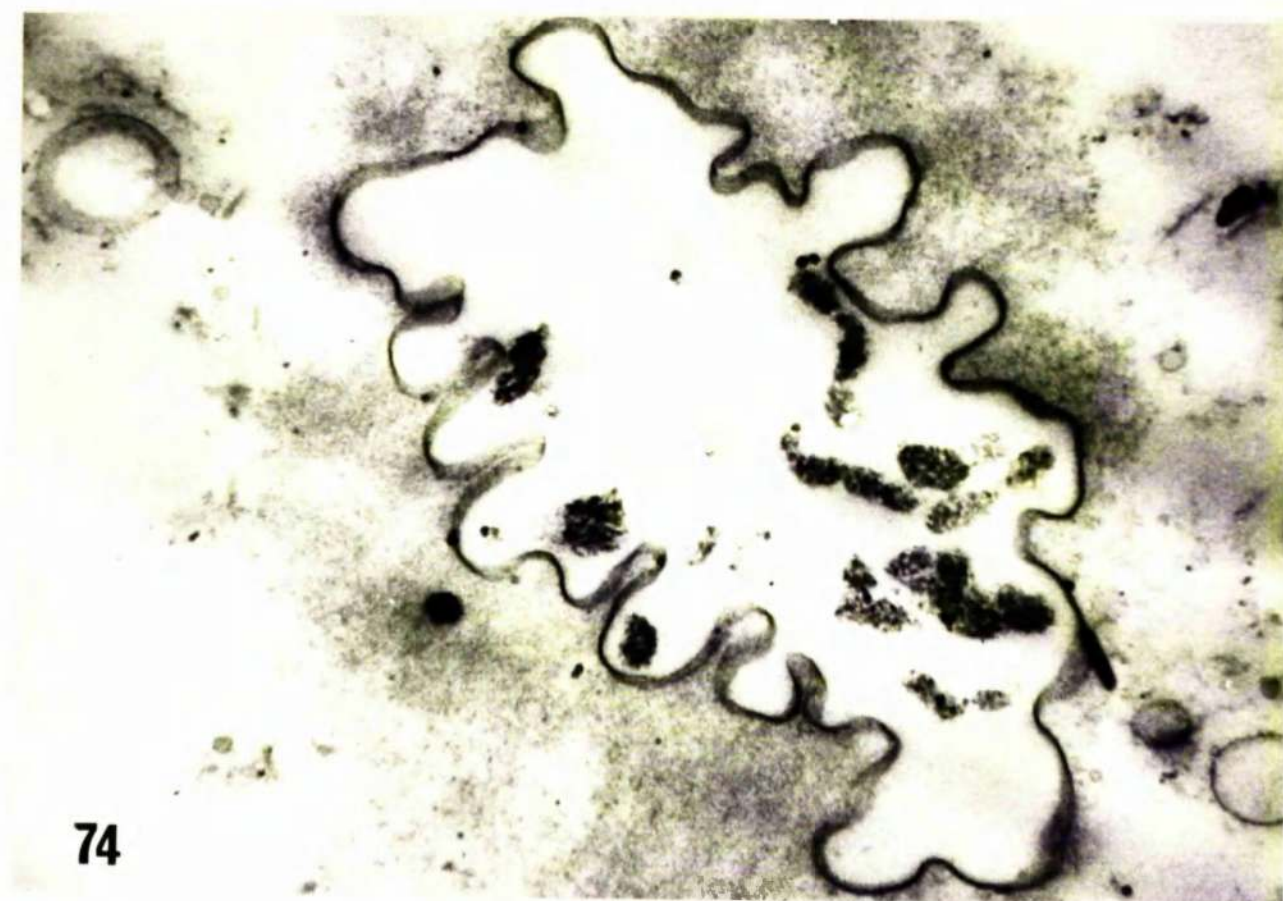
Micrograph 71 Photomontage of ten minute alcian blue induced channel (with amoeba surface to the left). There has been damage at the neck during preparation and the plasmalemma is broken. However, granular cytoplasm is stuck to the plasmalemma of the channel wall and separated from the remaining cytoplasm (broken line). There are some normal cell constituents near the channel and debris in the cytoplasm has accumulated at the edge of the granular collar. (mag. 7,500).



Micrograph 72 Tip of a developing channel. The
finger-like processes extend into densely
granulated cytoplasm. The lumen contains shed
abm as well as sundry ingested dense debris of
unknown origin. (mag. 30,000).



Micrographs 73 and 74 Cross sections of alcian
blue (ten and thirty minutes after immersion)
induced channels with small areas of granular
cytoplasm containing shed abm, clumped together
in 74 but remaining aligned in 73.
(mag. 73 - 12,500, 74 - 23,000).



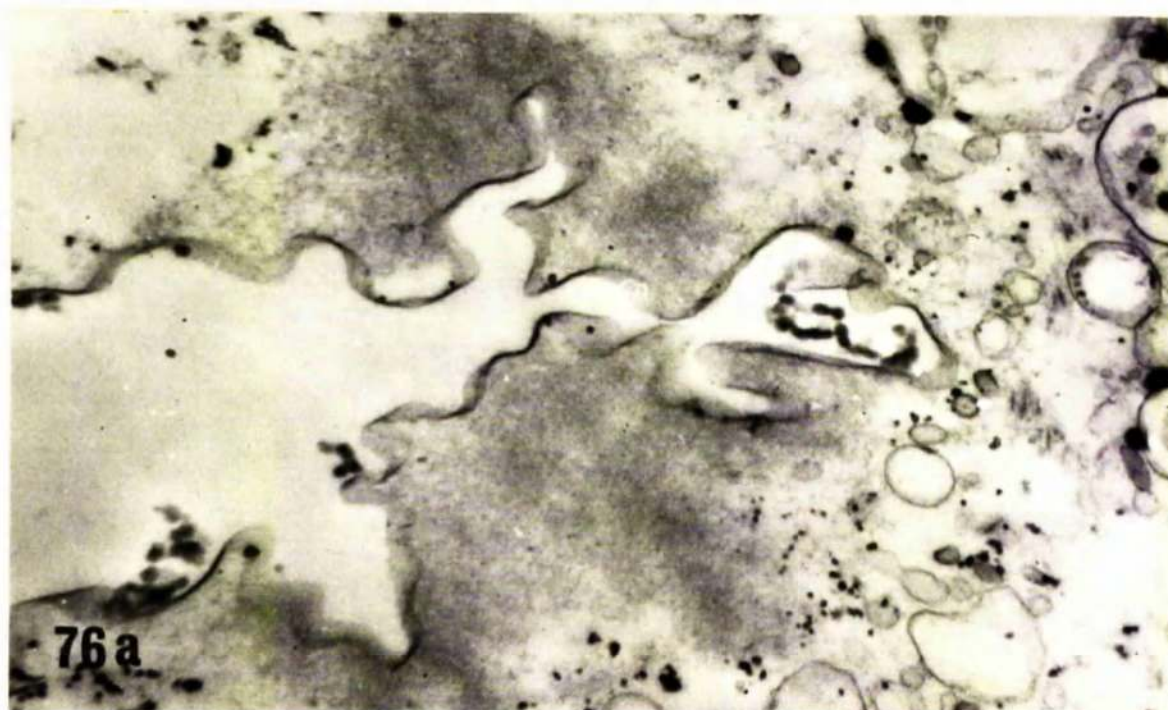
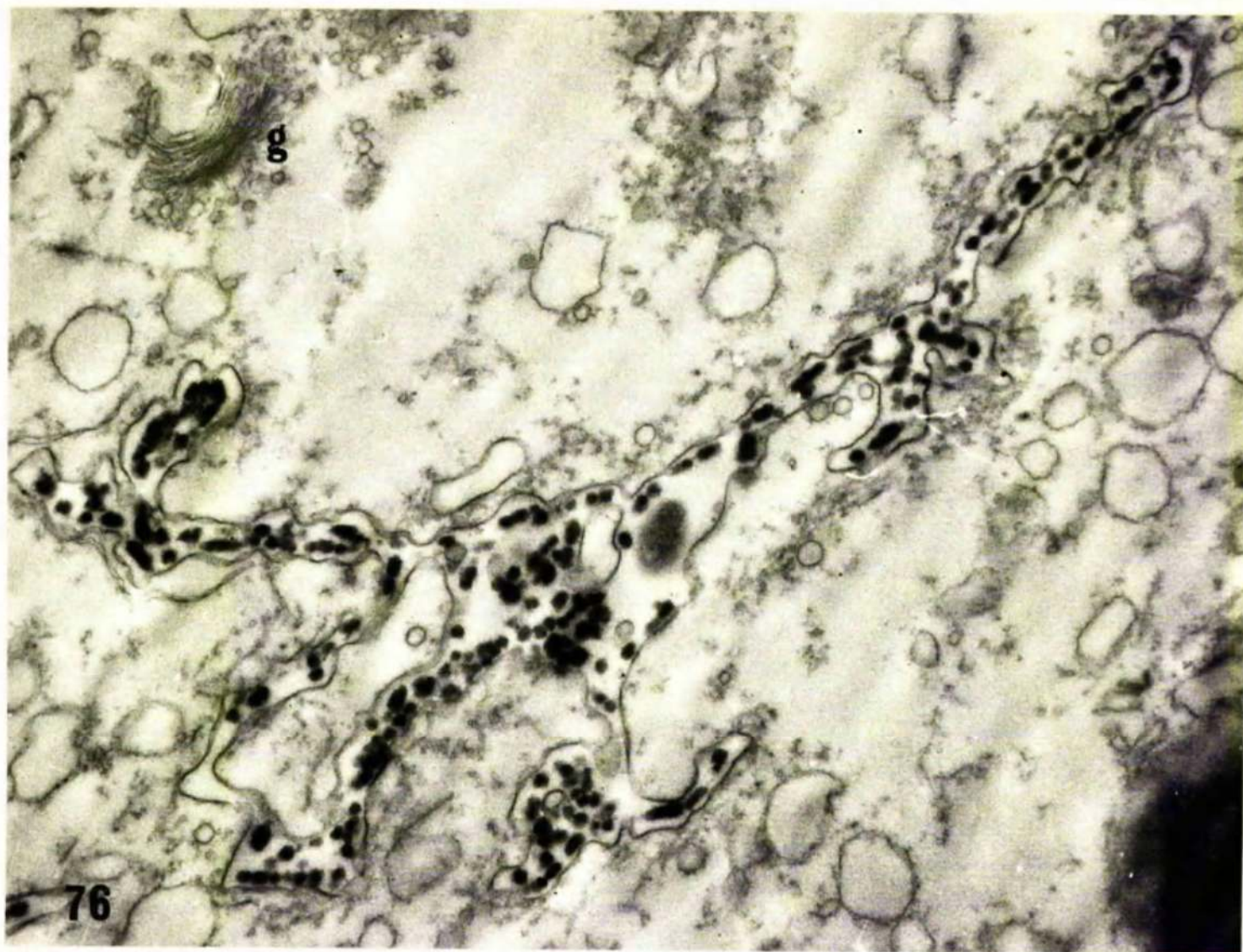
Micrograph 75 Developing channel thirty minutes after alcian blue. The lumen contains shed abm and is still connected to the outer surface at the neck (N). It is surrounded in part by granular cytoplasm through which the complex processes extend. There are a number of mitochondria in the vicinity. (mag. 11,000).

N



Micrograph 76 Channel fragment: after alcian blue treatment. It is a closed vesicle containing quantities of abm and no longer related to granular cytoplasm. (g = Golgi apparatus). (mag. 16,000).

Micrograph 76a Tip of advancing channel similar to that in 72. (mag. 18,000).

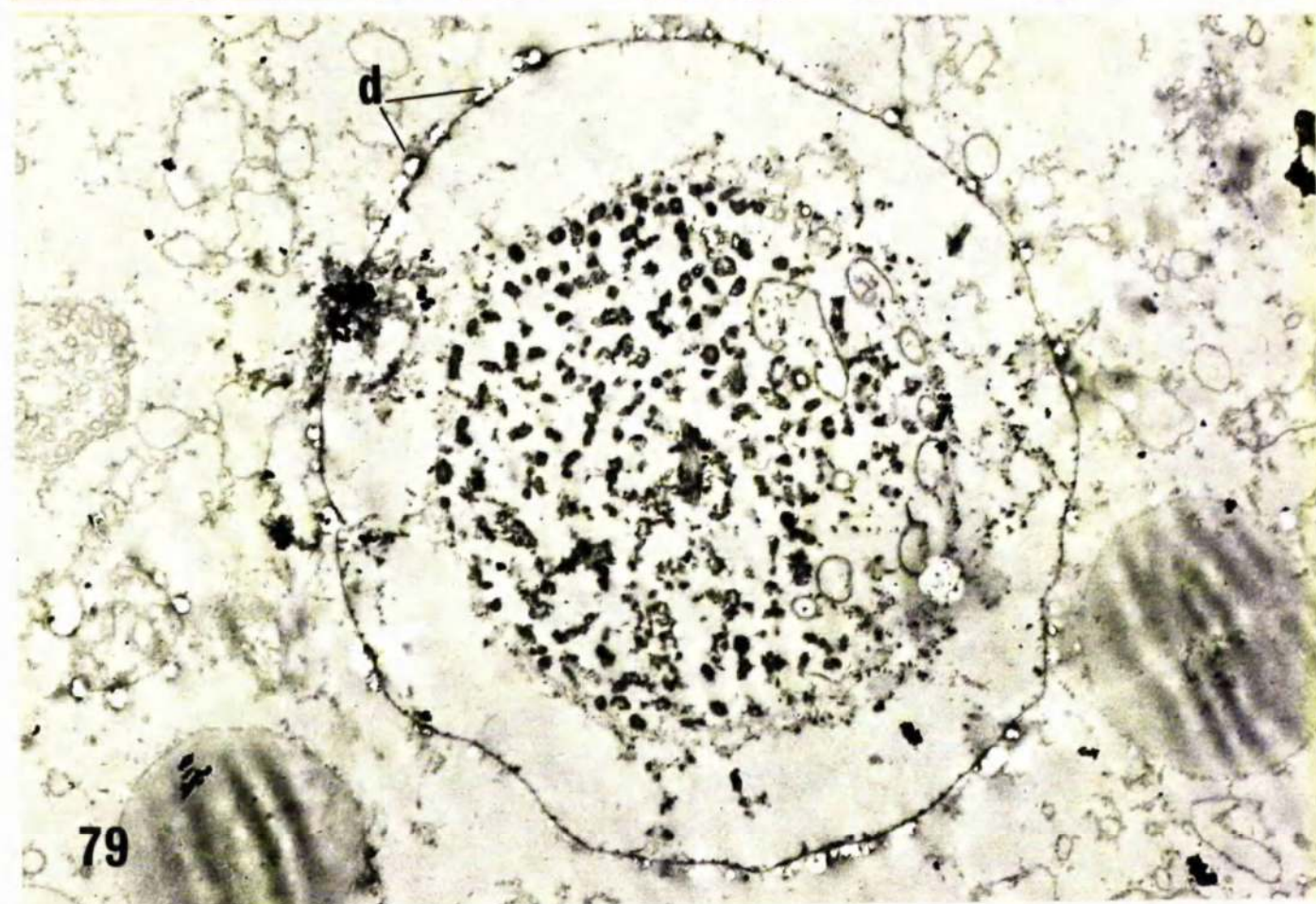
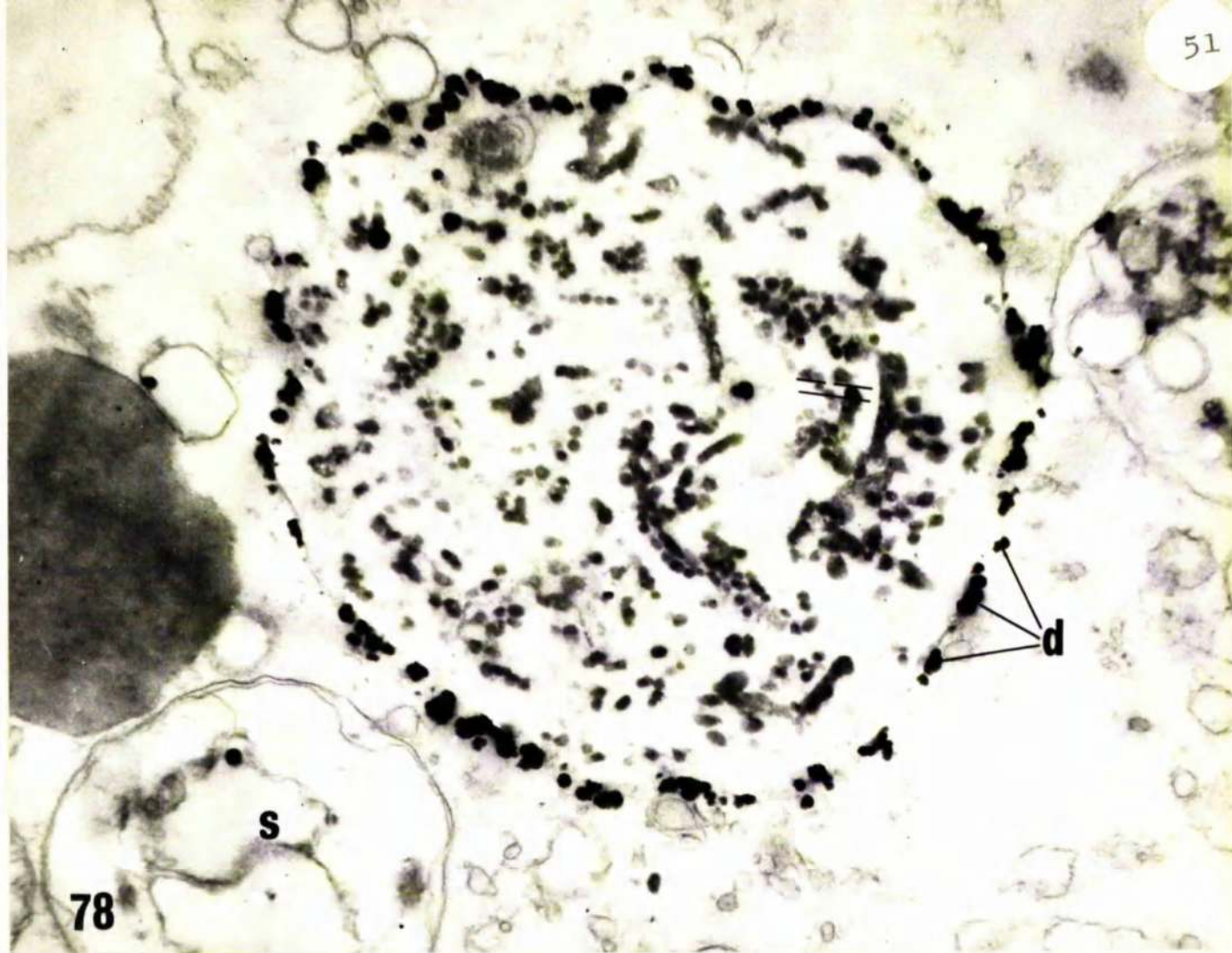


Micrograph 77 Beginning of a complex fusion droplet. In some unknown way a complete channel fragment containing abm has been incorporated into a droplet, the lumen of which also contains a small quantity of abm. The wall is studded with small dense bodies (d) in the way usually associated with plasmalemma or its derivatives. (mag. 20,000).

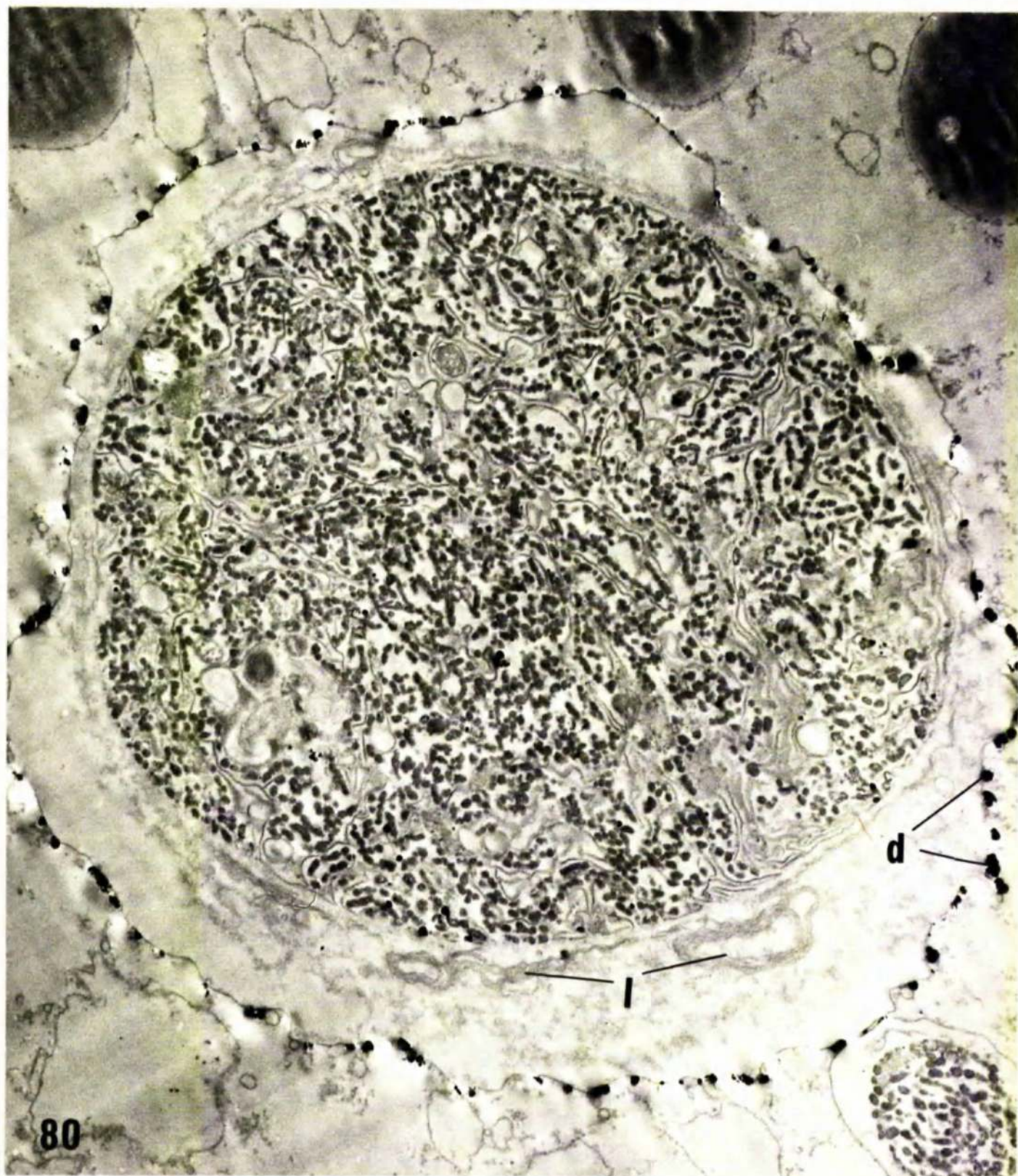


Micrograph 78 Fusion droplet containing only
abm some of which is still aligned as if
attached to the plasma membrane (==). The
limiting membrane is crowded with small dense
bodies (d). The structure in the cytoplasm
limited by two membranes (S) is a small
sequestration containing only a few membranes.
(Mag. 14,500).

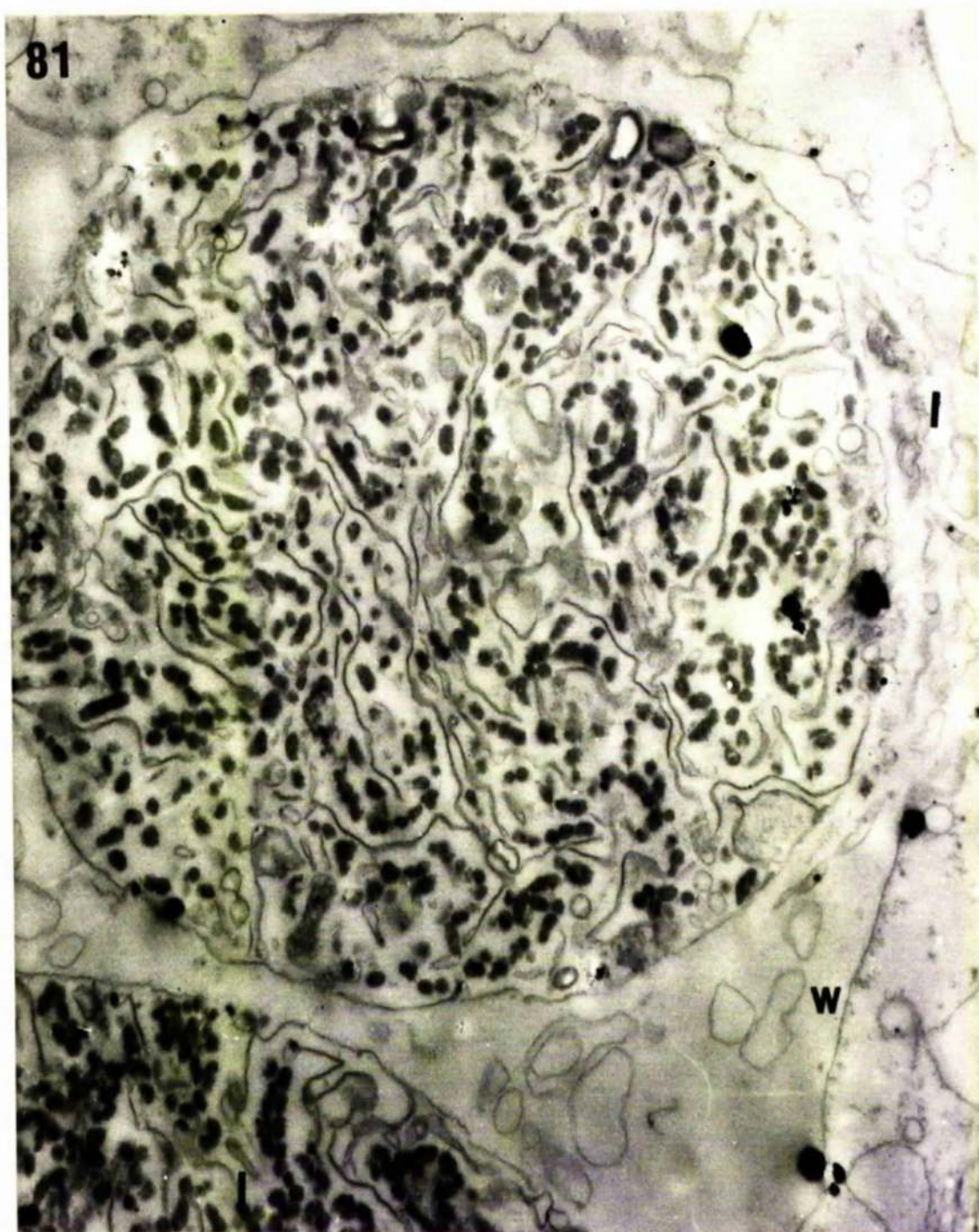
Micrograph 79 A structure like 78 stained with PTA
and printed at a low contrast. The cytoplasmic
constituents are unstained but the limiting
membrane of the droplet is moderately intensified.
The small dense bodies have completely disappeared
(d). (mag. 16,000).



Micrograph 80 Large complex fusion droplet containing abm and membrane compressed as a central spherical mass, which is separated from the outer membrane by a space. The space contains patchy granules and well marked lamellated annulae (1). The outer membrane is studded with small dense bodies (d). (mag. 11,000).

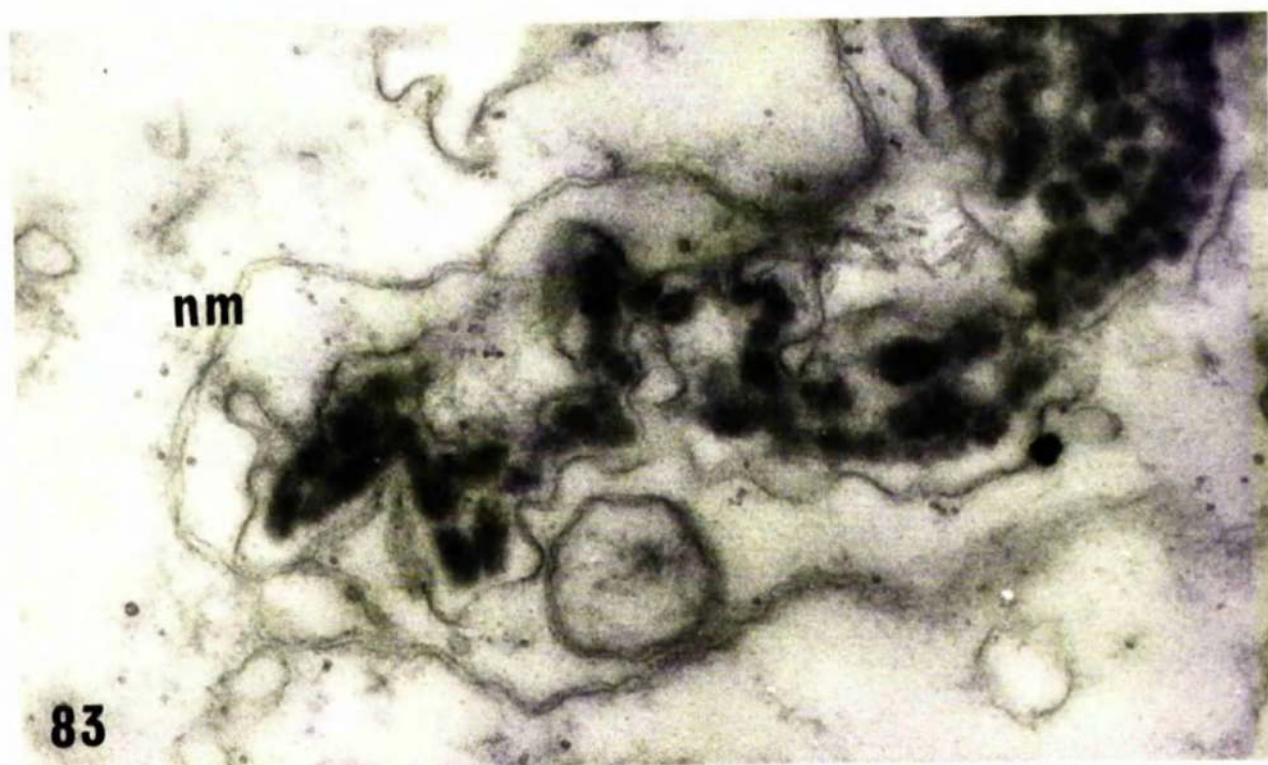
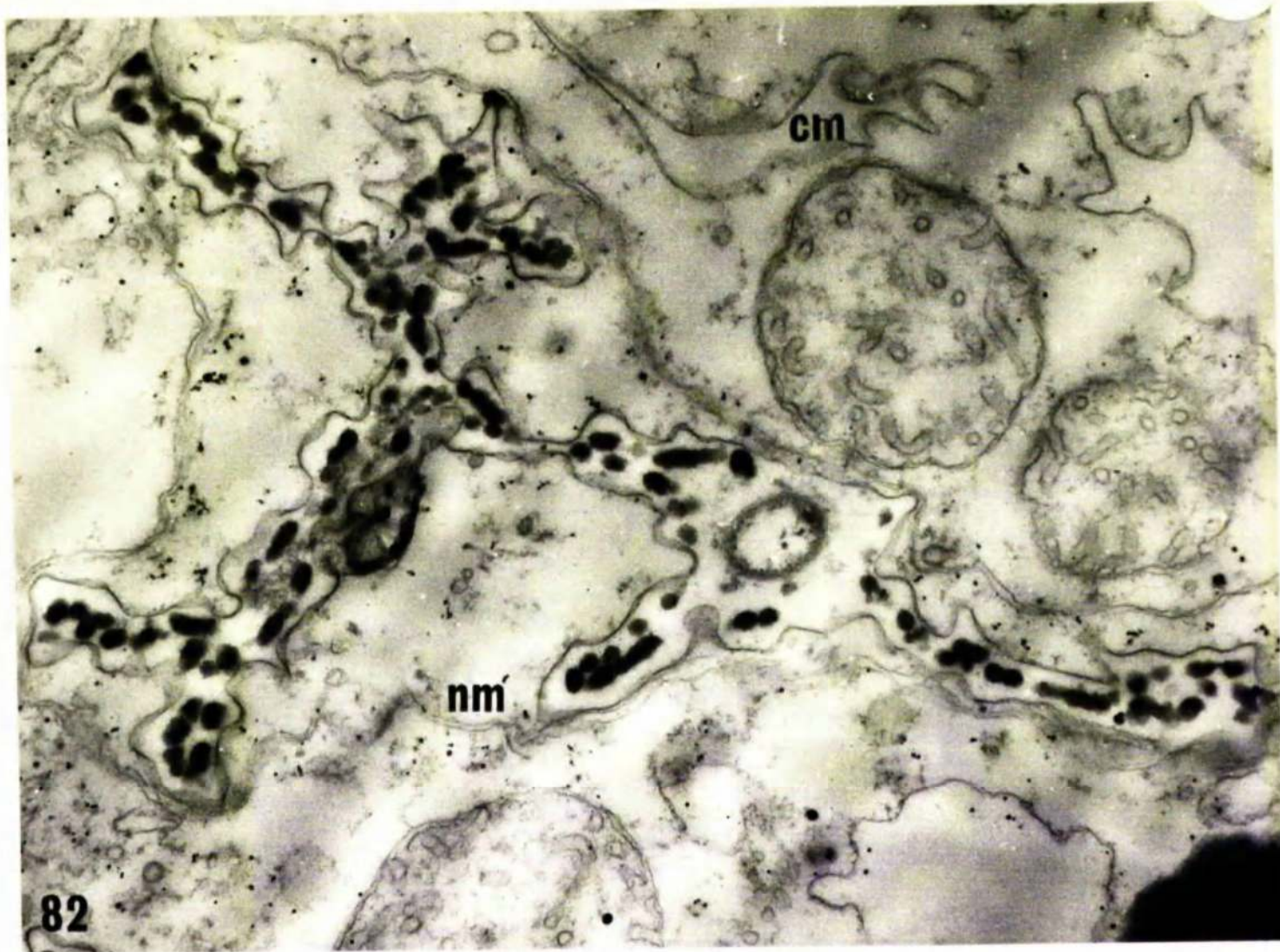


Micrograph 81 The fusion droplets such as the one in 80 fuse together but the central masses remain separate. In this case parts of two spherical masses can be seen and they are surrounded by a common outer membrane (w). Lamellated annulae are present in the peripheral space (1). (mag. 18,000).

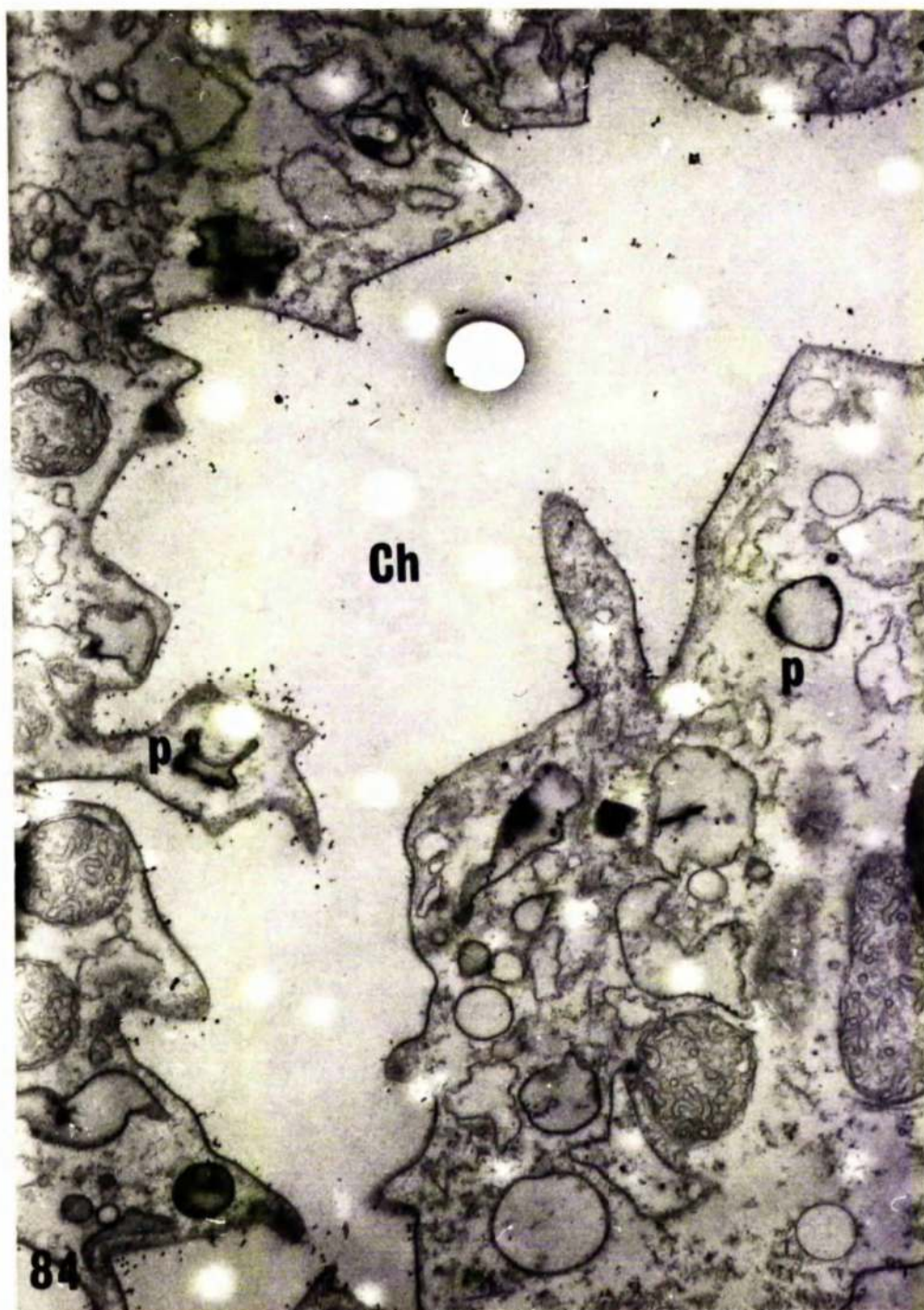


Micrograph 82 Four hours after intense induction channel fragments such as this are progressively surrounded by a new double membrane (nm) which in this case is complete. Its texture contrasts with that of the channel walls but resembles the cytomembranes (cm). (mag. 21,000).

Micrograph 83 A higher magnification of a similar channel with new surrounding membrane (nm). (mag. 25,000).

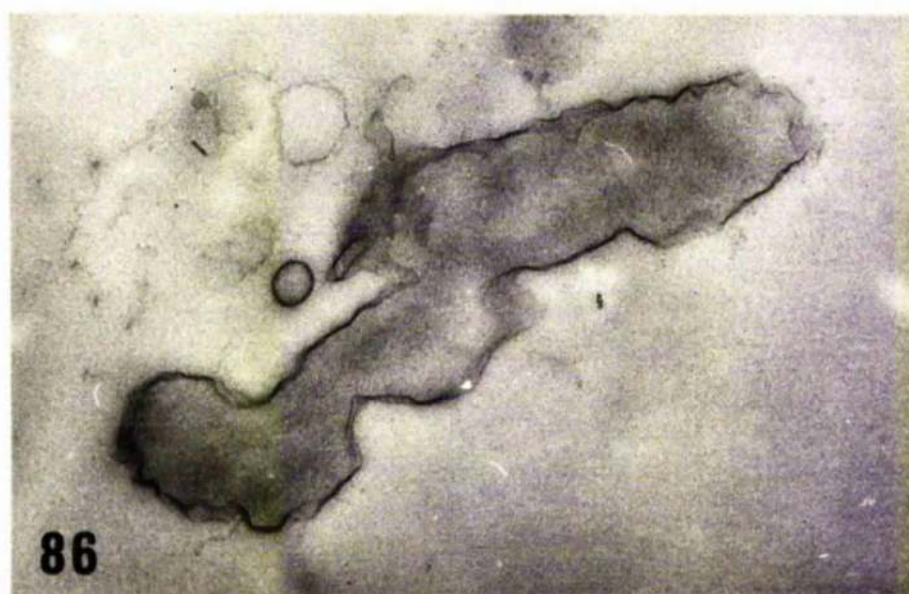
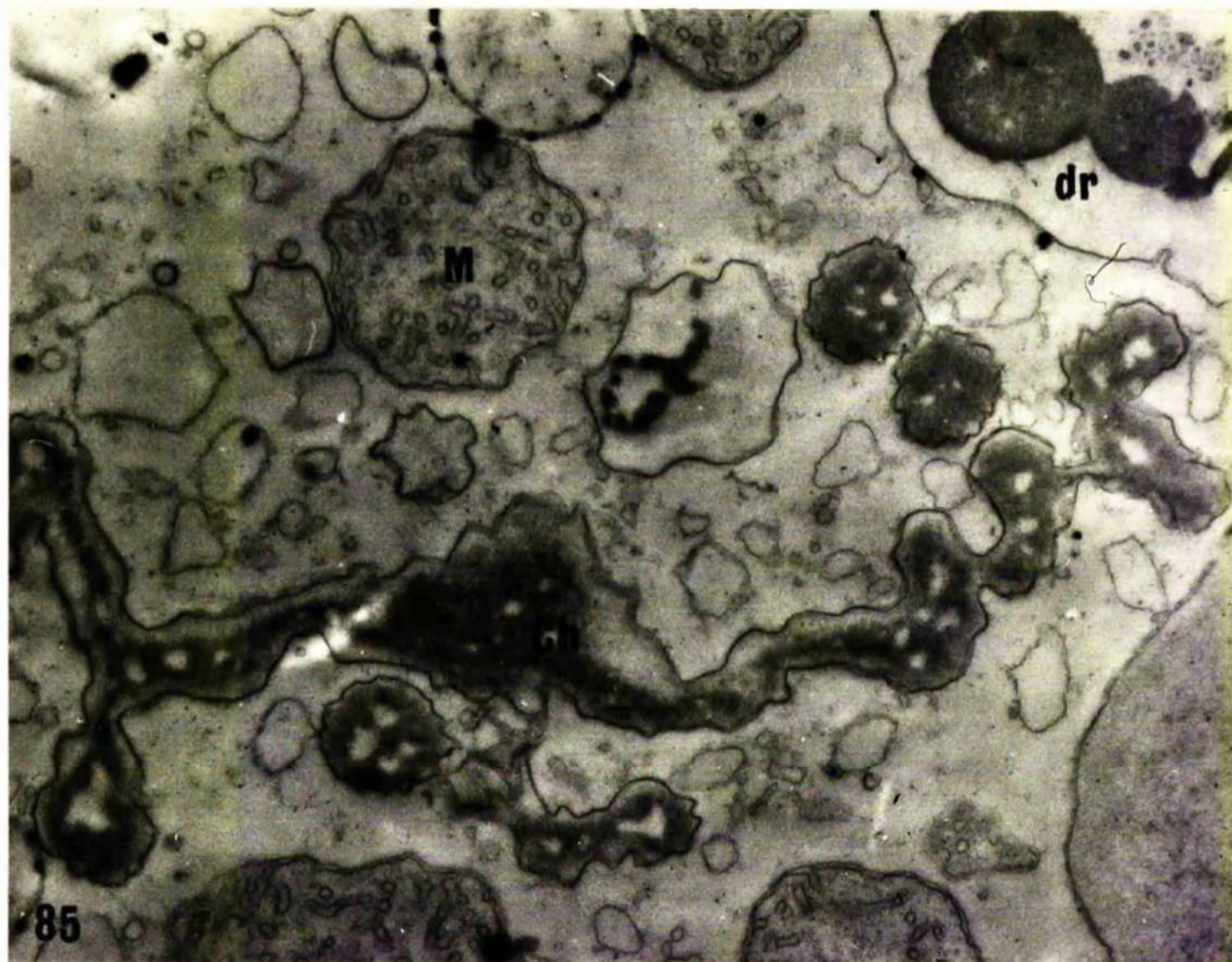


Micrograph 84 Pinocytosis channel following sodium chloride induction stained with PTA. Although in this case the plasmalemma of the channel proper (Ch) has not stained intensely, there are fragments of the filamentous layer visible. In the cytoplasm vesicles which would not otherwise be noticeable have stained selectively and presumably result from micropinocytosis (p). (mag. 12,000)



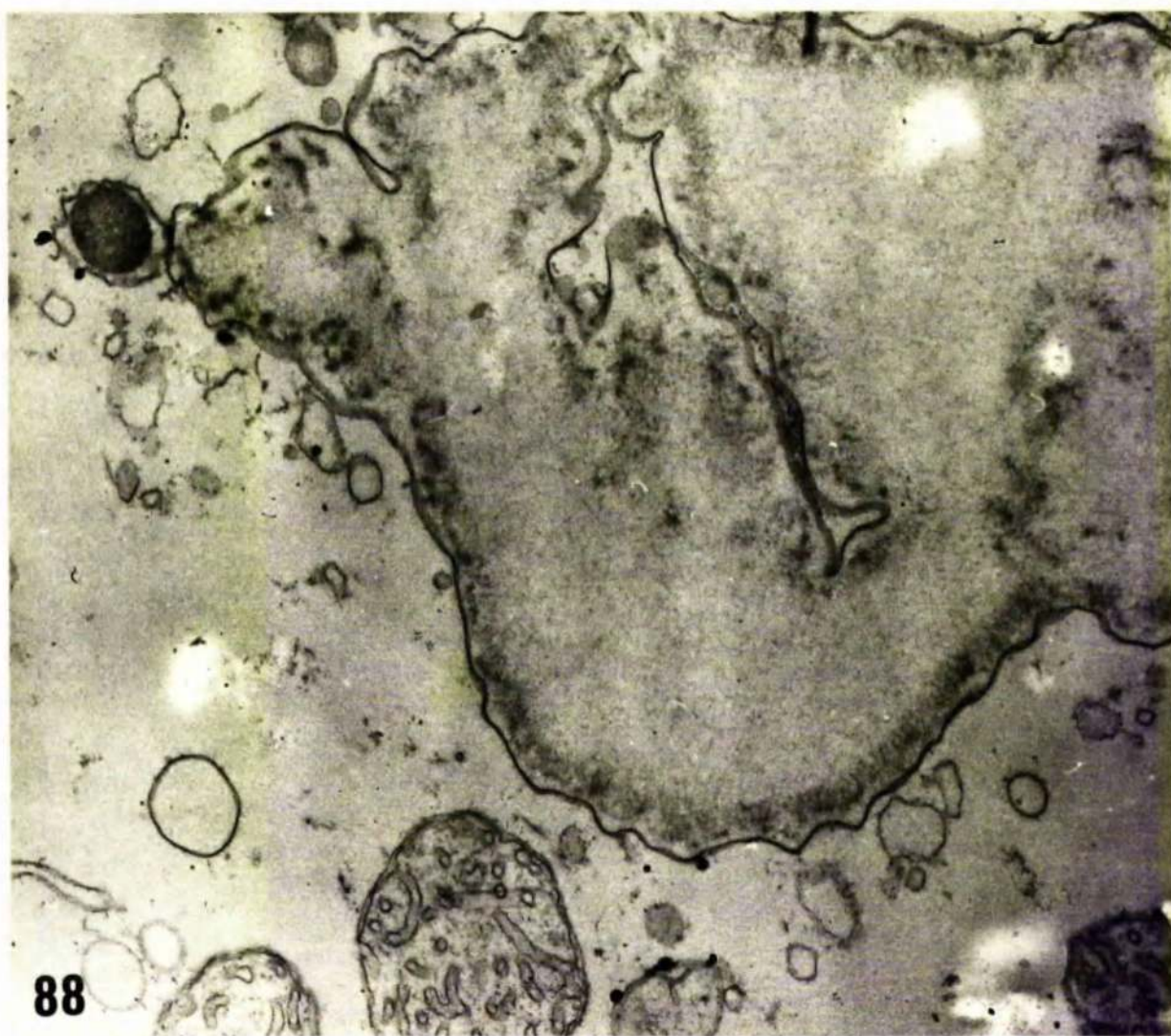
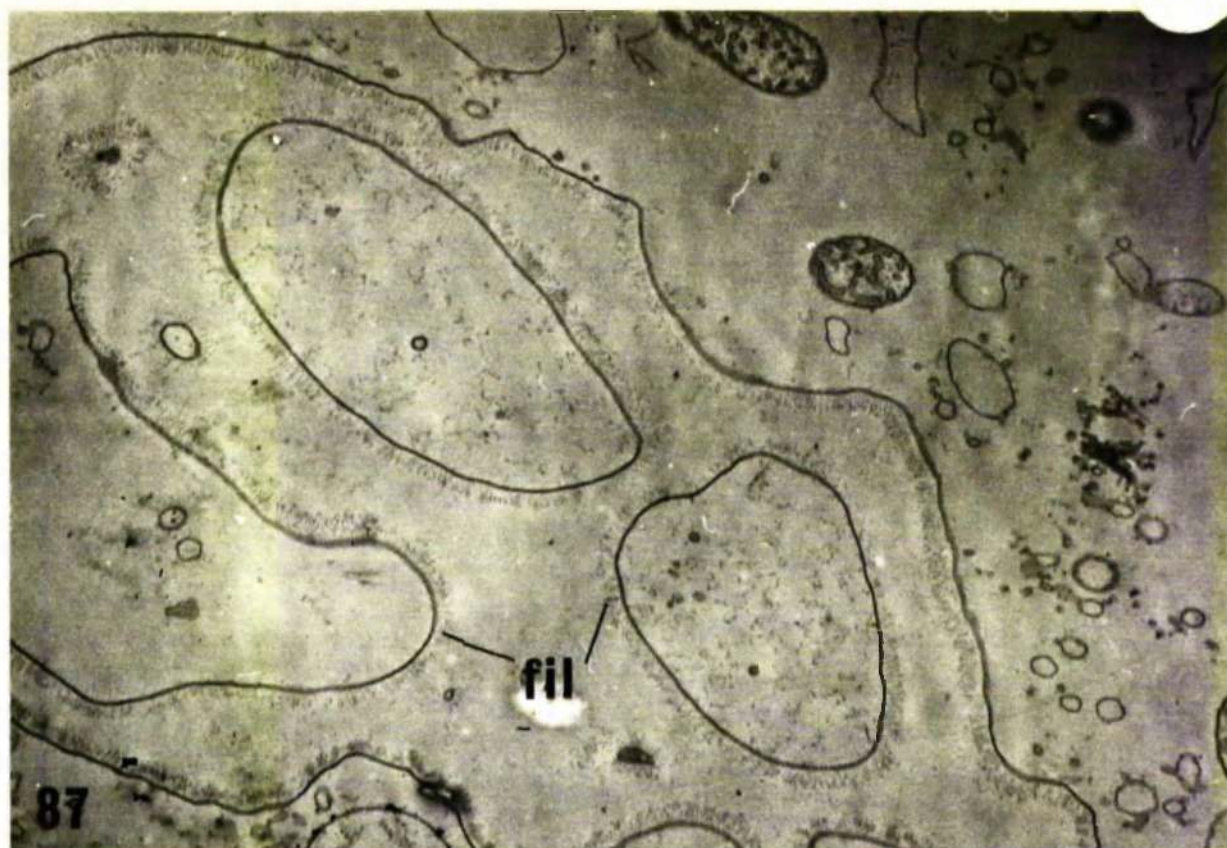
Micrograph 85 Channels resulting from induction of pinocytosis by bovine plasma albumin (Ch). There is a central amorphous mass consisting of protein and filament material. A fusion droplet (d) contains a rounded homogeneous mass of ingested material M - mitochondrion. (mag. 14,000).

Micrograph 86 Isolated fragment of channel from Albumin induction (five minutes) (mag. 19,000).



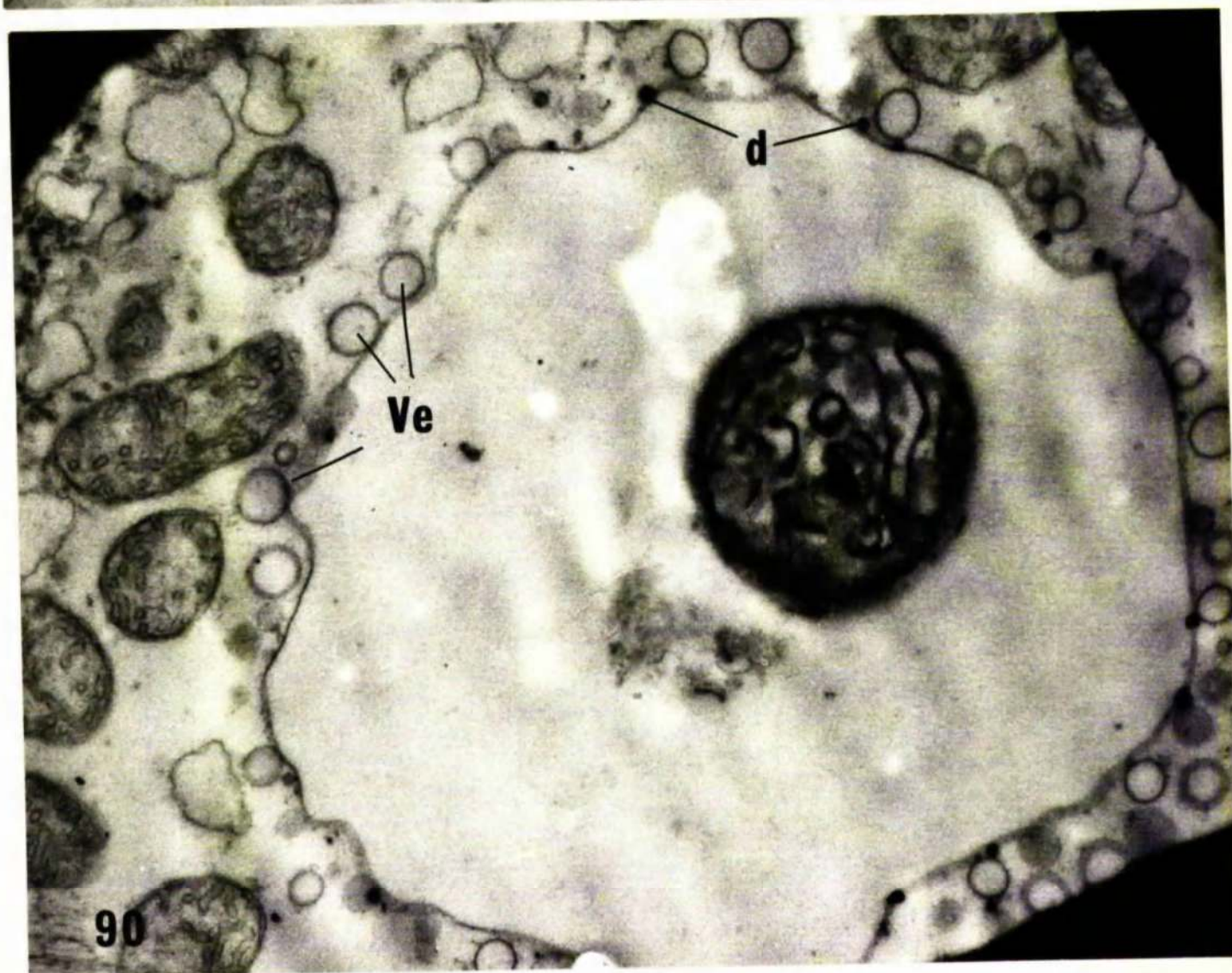
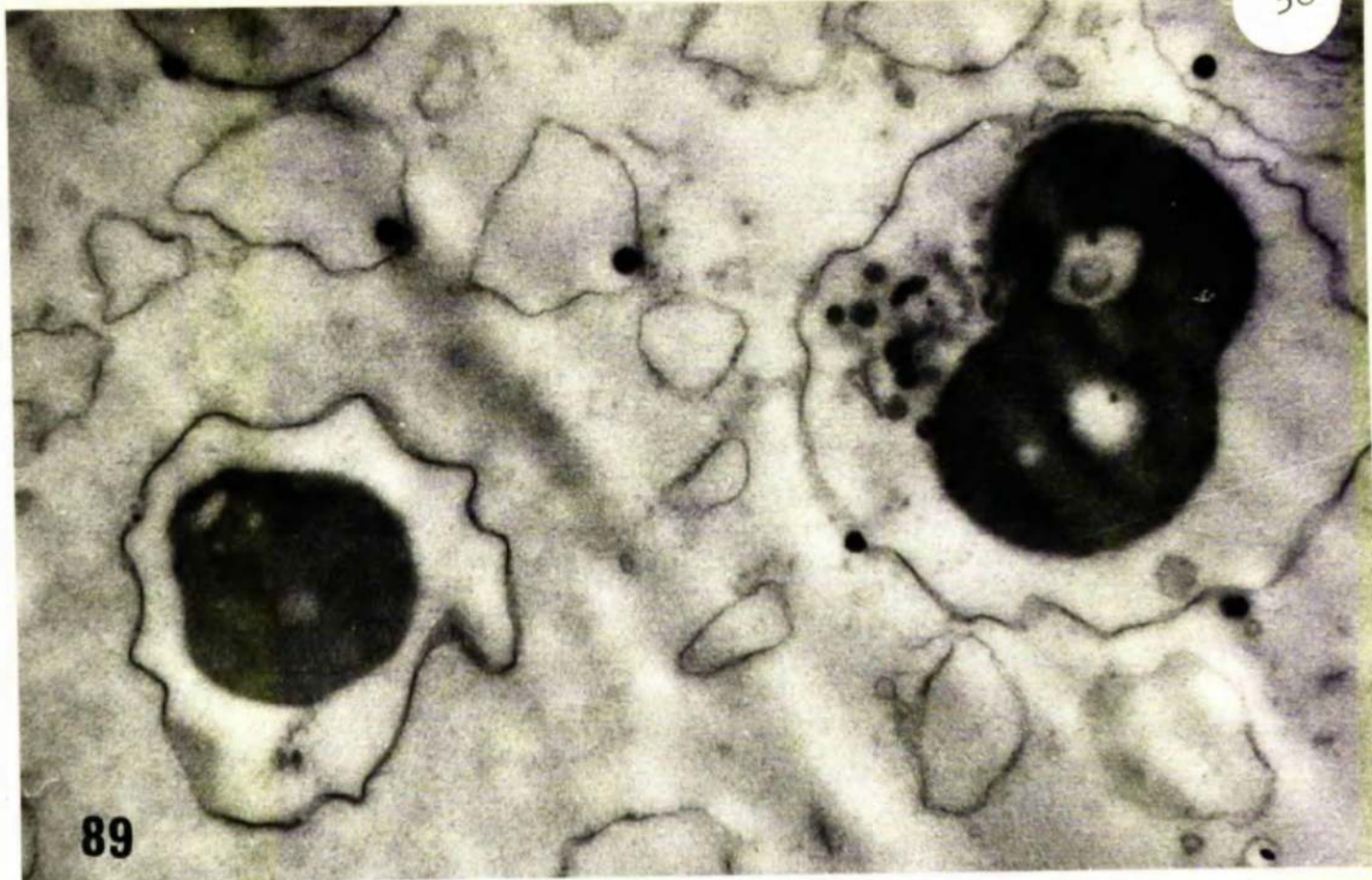
Micrograph 87 Early channel from albumin induction (five minutes). The filamentous part of the plasmalemma is visible (Fil) as parallel fibrils at right angles to the plasmalemma proper. (mag. 8,500).

Micrograph 88 Albumin channel (thirty minutes) there appears to be a heavier load of protein in this case and a loss of fine fibrillation of the filamentous layer. In both 87 and 88 there are attendant mitochondria though possibly no more than could occur in a random section of cytoplasm. (mag. 17,000).



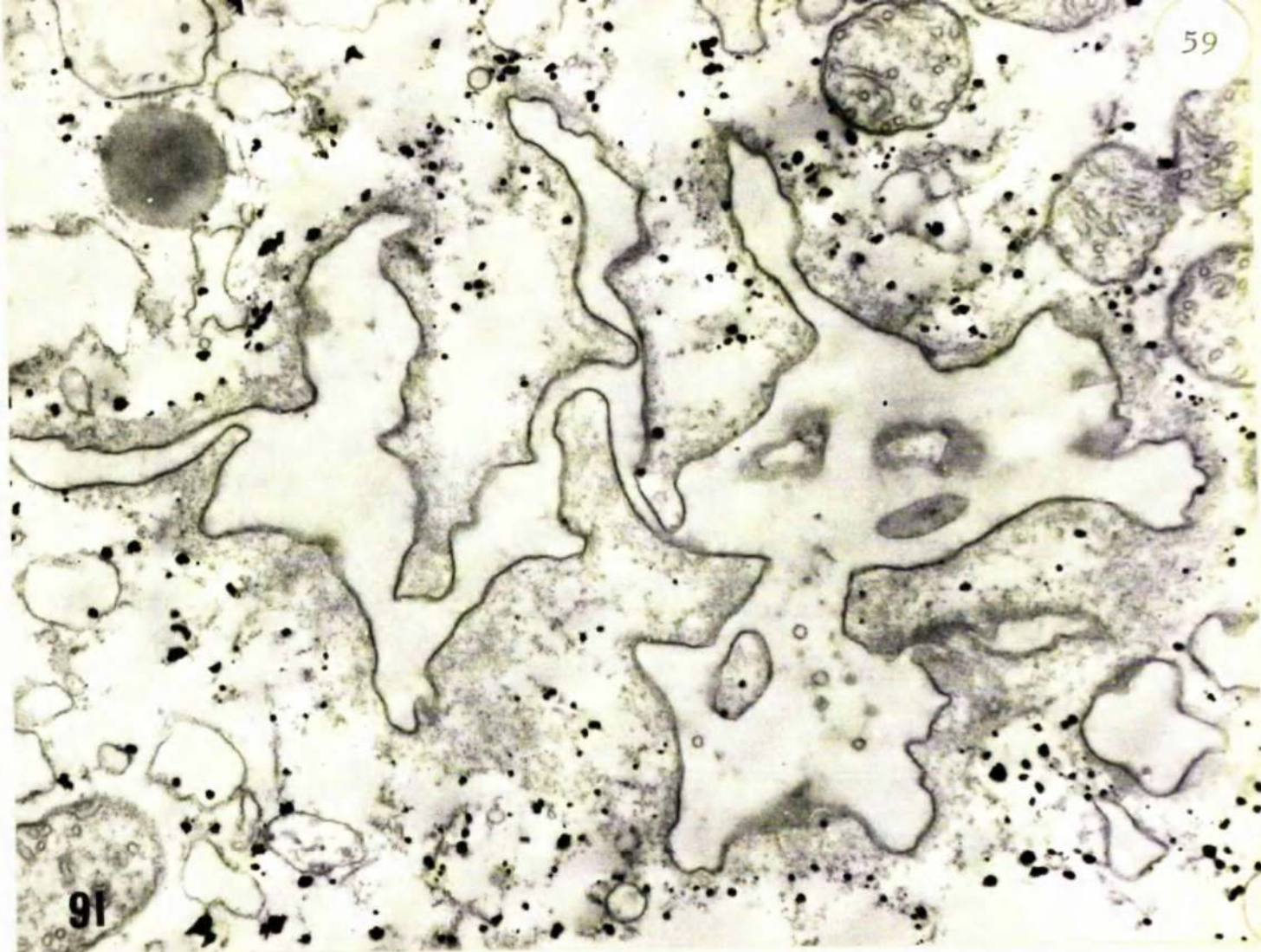
Micrograph 89 Fusion droplets of ingested albumin one hour after immersion with a densely osmiophilic central amorphous mass. The wall is distinctly denser than nearby cytomembranes. (mag. 15,000).

Micrograph 90 A fusion droplet one hour after sodium chloride induction. The contents compressed into a spherical ball consist entirely of dense membranous elements. The wall has a few small dense bodies (d) on its cytoplasmic surface and there are satellite vesicles (Ve). The cytoplasm contains mitochondria in unusually large numbers. Notice that this clearly resembles a food vacuole and cannot be certainly identified. (Mag. 13,000).



Micrograph 91 Channel fragment after albumin induction. This example contains no traces of filamentous material or protein and is surrounded by a narrow band of granular cytoplasm. The remaining cytoplasm contains large quantities of unidentified dense debris. (mag. 14,000).

Micrograph 92 Fusion droplet after alcian blue induction. The central mass contains membrane only and the peripheral space evidence of phospholipid in the form of lamellated annulae (1). (mag. 12,000).



91



92

Micrograph 93 Intense induction with alcian blue leads to toxic changes. In this case free lamellae (presumably of phospholipid) have appeared in the cytoplasm six hours after immersion for three minutes at pH 4.8. They are never seen in normal amoebae. The mitochondria (M) are abnormal. (mag. 28,000).

Micrograph 94 A similar body of material to 93 lying free and without a restricting membrane. Abnormal mitochondria and a residual channel (Ch) are also visible. (mag. 12,000).



Micrograph 95 A sequestration thirty minutes after
alcian blue induction. A fairly normal looking
mitochondrion is completely trapped within two
concentric membranes of which the inner is pleated.
The normal structure of the mitochondrion might be
related to the large amount of cytoplasm
incorporated in this case. (mag. 32,000).

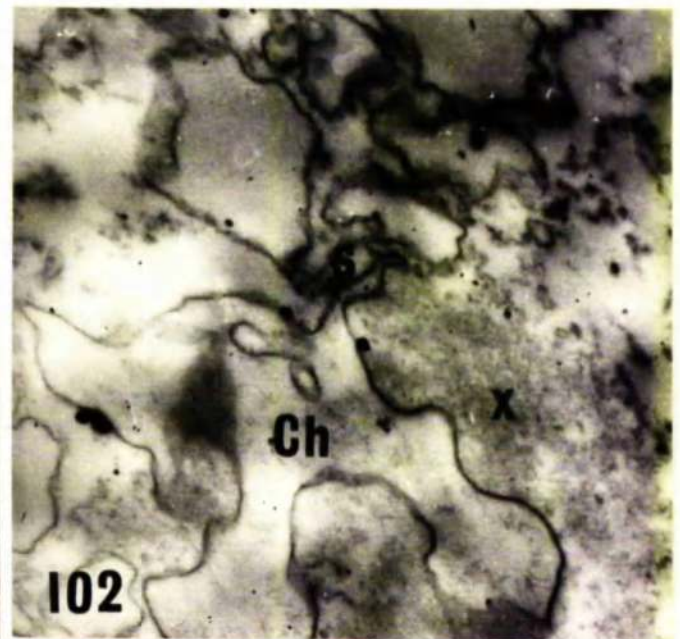
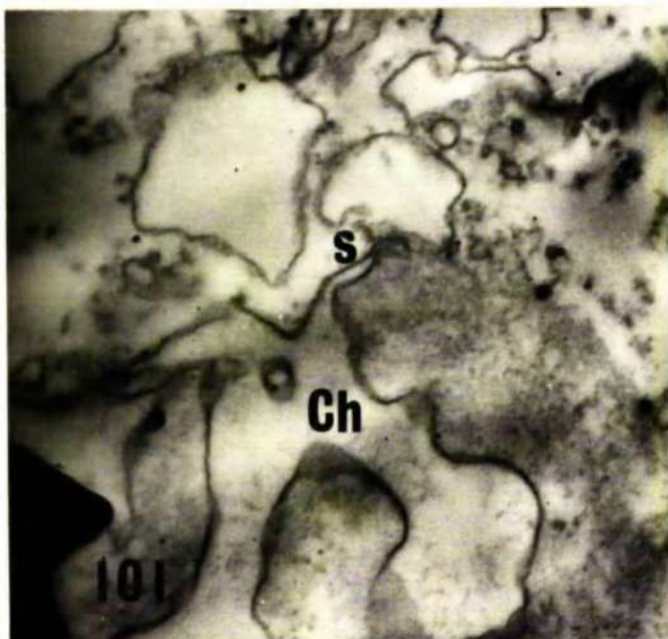
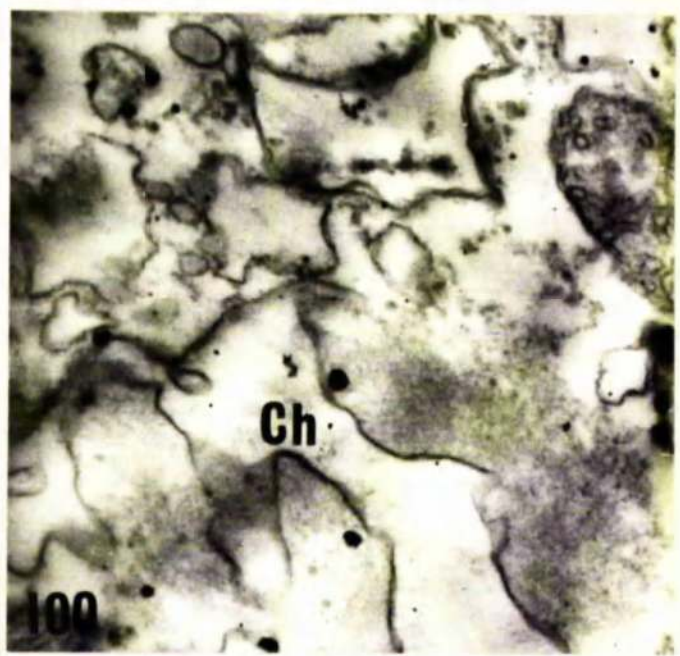
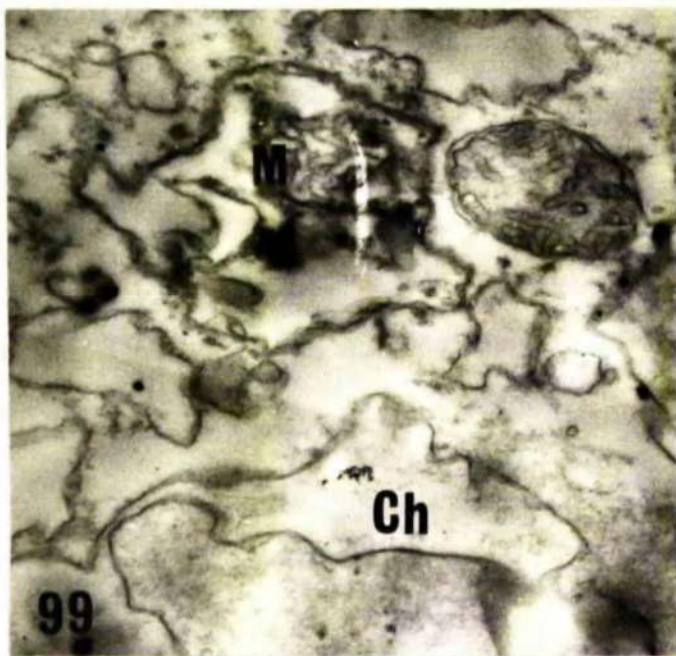
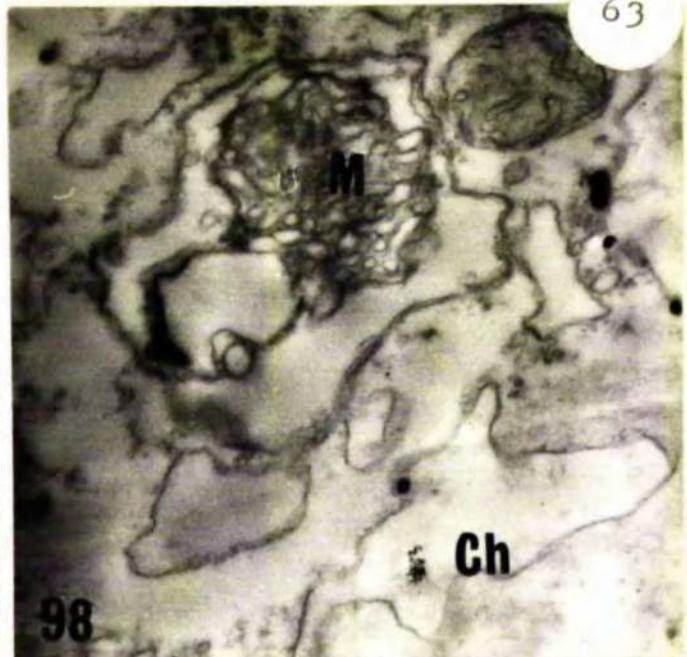
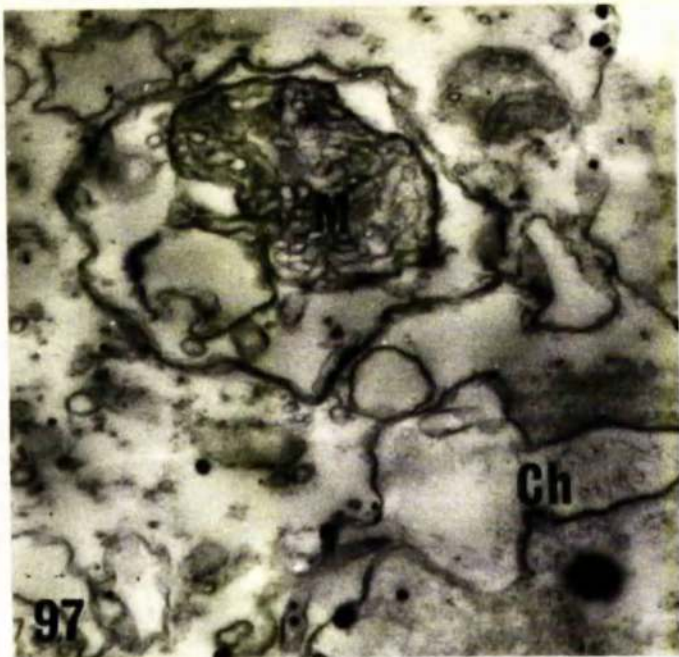


Micrograph 96 A more complex sequestration two hours after alcian blue containing a mitochondrion (m) with densely crowded tubules and increased density, a fat droplet (F) and cytoplasmic membranes possibly part of the Golgi apparatus (g), all surrounded by two membranes. This is a poor section but such phenomena are met only by chance and not always in good sections. (mag. 32,000).



Micrographs 97 - 102 Serial sections from an amoeba ten minutes after sodium chloride immersion at the peak of channel formation which by chance contained a sequestration and suggested it's mode of formation from a channel.

The channel (Ch) contains filamentous material to an unusual degree for a sodium chloride preparation. A grossly altered mitochondrion (M), compared with the normal one lying nearby, is enclosed by two distinct membranes. The channel lumen seems to be in continuity at "S" with the space between the two enclosing membranes of the sequestration. Note the granular cytoplasm related to the channel (X). (mag. 18,000).



Micrograph 103 Golgi apparatus two hours after
induction of pinocytosis with alcian blue.

The flattened sacs or double membranes become
dilated towards the apex until they seem to
consist of interconnected small round vesicles
which are very similar to the free round
vesicles nearby in the cytoplasm. (mag. 45,000).

